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[001] ELECTROCHEMICAL DETECTION SEQUENCE-SPECIFIC NUCLEIC ACID OLIGOMER HYBRIDIZATION EVENTS

[002] FIELD OF THE INVENTION

[003] The present invention is directed to a modified nucleic acid oligomer, as well as a method of electrochemically detecting sequence-specific nucleic acid oligomer hybridization events.

[004] BACKGROUND OF THE INVENTION

[005] Generally, gel-electrophoretic methods with autoradiographical or optical detection are used for DNA and RNA sequence analysis, for example in disease diagnosis, toxicological test procedures, genetic research and development, and the agricultural and pharmaceutical sectors.

[006] In the most significant gel-electrophoretic method with optical detection, the Sanger method, a solution containing DNA is divided into four samples. To differentiate the four samples, the primer (complementary starting sequence for replication) of each sample is covalently modified with a fluorescent dye that emits at a distinct wavelength. Starting at the primer, each sample is enzymatically replicated by DNA polymerase I. In addition to the requisite deoxyribonucleoside triphosphates of the bases A (adenine), T (thymine), C (cytosine), and G (guanine), each reaction mixture also contains sufficient 2',3'-dideoxy analog of one of these nucleoside triphosphates as a blocking base (one of each of the four possible blocking bases per sample) in order to terminate replication at all possible binding sites. After the four samples are combined, all lengths of replicated DNA fragments having blocking-base-specific fluorescence result and can be sorted gel-electrophoretically by length and characterized by fluorescent spectroscopy.

[007] Another optical detection method is based on the attachment of fluorescent dyes such as ethidium bromide on oligonucleotides. In comparison with a free solution of the dye, the fluorescence of such dyes changes drastically upon association with double-stranded DNA or RNA and can therefore be used to detect hybridized DNA or RNA.

[008] In radiolabeling, ^{32}P is built into the phosphate skeleton of the oligonucleotides, ^{32}P usually being added to the 5'-hydroxyl end by polynucleotide

[010] In addition to what is known as de novo sequencing of as yet unknown oligonucleotide sequences, oligonucleotide sequences or DNA fragments that code one or more known genes also can be bound to the oligomer chip described above. Thus, for example, for each gene being sought and having a known base sequence, a sufficient number of oligonucleotide sequences comprising, for example, 20 bases each and being complementary to appropriate sequence sections of the gene being sought and having a known base sequence can be applied to a support material in order to detect this gene with very high probability. On the other hand, on an oligomer chip, known genes, too, can be tested for mutations, for example by applying to the support material the appropriate sequence sections of the genes with and without mutations.

[011] The use of radioactive labels in DNA/RNA sequencing is associated with several disadvantages, such as elaborate, legally required safety precautions in dealing with radioactive materials, radiation exposure, limited spatial resolution capacity (maximum 1 mm^2), and sensitivity that is high only when the radiation of the radioactive fragments act on an X-ray film for an appropriately long time (hours to days). Although the spatial resolution can be increased by additional hardware and software, and the detection time can be decreased by using beta scanners, both of these involve considerable additional costs.

[012] Some of the fluorescent dyes that are commonly used to label the DNA (e.g., ethidium bromide) are mutagenic and require appropriate safety precautions, as does the use of autoradiography. In nearly every case, the use of optical detection requires the use of one or more laser systems, and thus experienced personnel and appropriate safety precautions. The actual detection of the fluorescence requires additional hardware, such as optical components for amplification and, in the case of varying excitation and query wavelengths as in the Sanger method, a control system. Thus, depending on the required excitation wavelengths and the desired detection performance, considerable investment costs may result. In sequencing by hybridization on an oligomer chip, detection is even more costly since, in addition to the excitation system, high-resolution CCD cameras (charge coupled device cameras) are needed for the two-dimensional detection of fluorescent spots.

[013] Thus, although quantitative and extremely sensitive methods for DNA/RNA sequencing exist, these methods are time consuming, require painstaking sample preparation and expensive equipment, and are generally not available as portable systems.

[014] DESCRIPTION OF THE INVENTION

[015] Therefore, it is the object of the present invention to create for detecting nucleic acid oligomer hybrids an apparatus and a method that do not exhibit the disadvantages of the background art.

[016] According to the present invention, this object is solved by the modified nucleic acid oligomer according to independent claim 1, the method of producing a modified nucleic acid oligomer according to independent claim 22, the modified conductive surface according to independent claim 34, the method of producing a modified conductive surface according to independent claim 49, and a method of electrochemically detecting nucleic acid oligomer hybridization events according to independent claim 53.

[017] The following abbreviations and terms will be used in the context of the present invention:

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PNA	Peptide nucleic acid (synthetic DNA or RNA in which the sugar-phosphate moiety is replaced by an amino acid. If the sugar-phosphate moiety is replaced by the -NH-$(CH_2)_2$-N($COCH_2$-base)-CH_2CO- moiety, PNA will hybridize with DANN.)
A	Adenine
G	Guanine
C	Cytosine
T	Thymine
U	uracil
base	A, G, T, C, or U

kinase. Thereafter, the labeled DNA is preferably cleaved, under defined conditions, at one of each of the four nucleotide types, such that an average of one cleavage per chain results. Thus, for a given base type, there are present in the reaction mixture chains extending from the ^{32}P-label to the position of that base (if there are multiple appearances of the base, chains of varying lengths will result accordingly). The four fragment mixtures are then gel-electrophoretically separated on four lanes. Thereafter, an autoradiogram of the gel is prepared, from which the sequence can be read directly.

[009] Some years ago, a further method of DNA sequencing was developed on the basis of optical (or autoradiographical) detection, namely sequencing by oligomer hybridization (cf. e.g. Drmanac et al., Genomics 4, (1989), pp. 114-128 or Bains et al., Theor. Biol. 135, (1988), pp. 303-307). In this method, a complete set of short oligonucleotides or nucleic acid oligomers (probe oligonucleotides), e.g. all 65,536 possible combinations of bases A, T, C, and G of an oligonucleotide octamer, are bound to a support material. The attachment occurs in an ordered grid comprising 65,536 test sites, each rather large amount of an oligonucleotide combination defining one test site, and the position of each individual test site (oligonucleotide combination) being known. On such a hybridization matrix, the oligomer chip, a DNA fragment whose sequence is to be determined (the target) is labeled with fluorescent dye (or ^{32}P) and hybridized under conditions that allow only one specific double-strand formation. In this way, the target DNA fragment binds only to those nucleic acid oligomers (in this example to the octamers) whose complementary sequence corresponds exactly to a portion (an octamer) of its own sequence. Thus, all of the nucleic acid oligomer sequences (octamer sequences) present in the fragment are determined by optical (or autoradiographical) detection of the binding position of the hybridized DNA fragment. Due to the overlapping of neighboring nucleic acid oligomer sequences, the sequential sequence of the DNA fragment can be determined using appropriate mathematical algorithms. One of the advantages of this method lies in the miniaturization of the sequencing and thus in the enormous amount of data that can be simultaneously captured in one operation. In addition, primer and gel-electrophoretic separation of the DNA fragments can be dispensed with. This principle is exemplified in Fig. 1 for a 13-base-long DNA fragment.

redox-active moiety	Equivalent to a catalytically redox-active moiety.
catalytically redox-active moiety	In the context of the present invention, a moiety referred to using the generic term "catalytically redox-active moiety" usually consists of one or more redox-active centers (cofactors, prosthetic groups), which are referred to in the following as electron donors or electron acceptors, and one or more macromolecules binding these redox-active centers. Thus, in its form that is relevant to the present invention, the catalytically redox-active moiety includes one or more electron-donor molecules and/or one or more electron-acceptor molecules, this (these) electron-donor molecule(s) and/or this (these) electron-acceptor molecule(s) being/becoming bound to one or more macromolecules or being embedded in this (these) macromolecule(s). Electron donor(s) and/or electron-acceptor(s) may be joined with one another via one or more covalent or ionic bonds, via hydrogen bonds, van der Waals bridges, π-π-interaction, or via coordination by means of electron-pair donation and acceptance, covalent links being able to be direct or indirect (e.g. via a spacer, but not via a nucleic acid oligomer) links. In addition, the electron donor(s) and/or electron acceptor(s) may be joined with the macromolecule(s) via covalent attachment to the macromolecule(s), via encapsulation in suitable molecular cavities (binding pockets) of the macromolecule(s), via ionic bonds, hydrogen bonds, van der Waals bridges, π-π-interaction, or via coordination by means of electron-pair donation and acceptance between the macromolecule(s) and the electron-donor molecule(s) and/or electron-acceptor molecule(s). If the catalytically redox-active moiety is composed of multiple macromolecules, the binding of the macromolecules to one another may likewise take place covalently, ionically, via hydrogen bonds, van der Waals bridges,

bp	base pair
nucleic acid	At least two covalently-joined nucleotides or at least two covalently-joined pyrimidine (e.g. cytosine, thymine, or uracil) or purine bases (e.g. adenine or guanine). The term nucleic acid refers to any backbone of the covalently-linked pyrimidine or purine bases, such as the sugar-phosphate backbone of DNA, cDNA, or RNA, a peptide backbone of PNA, or analogous structures (e.g. a phosphoramide, thiophosphate, or dithiophosphate backbone). The essential feature of a nucleic acid according to the present invention is that it can sequence-specifically bind naturally occurring cDNA or RNA.
nucleic acid oligomer	Nucleic acid of a base length that is not further specified (e.g. nucleic acid octamer: a nucleic acid having any backbone in which 8 pyrimidine or purine bases are covalently bound to one another).
oligomer	Equivalent to nucleic acid oligomer.
oligonucleotide	Equivalent to oligomer or nucleic acid oligomer, e.g. a DNA, PNA, or RNA fragment of a base length that is not further specified.
oligo	Abbreviation for oligonucleotide.
primer	Initial complementary fragment of an oligonucleotide, the base length of the primer being only approx. 4-8 bases. Serves as the starting point for enzymatic replication of the oligonucleotide.
mismatch	To form the Watson-Crick double-stranded oligonucleotide structure, the two single-strands hybridize in such a way that the A (or C) base of one strand forms hydrogen bonds with the T (or G) base of the other strand (in RNA, T is replaced by uracil). Any other base pairing does not form hydrogen bonds, distorts the structure and is referred to as a "mismatch".
ss	single-strand
ds	double-strand

electron acceptor is a molecule that can take up an electron from an electron donor, directly or under the influence of certain external conditions. For example, one such external condition is the oxidation or reduction of the electron donor or acceptor of the catalytically redox-active moiety by an external oxidizing or reducing agent, so for example the transfer of an electron to the electron donor by a reducing agent, or the release of an electron by the electron acceptor to an oxidizing agent. These oxidizing or reducing agents may be external redox-active substances, i.e. they are not covalently joined with but in contact with the catalytically redox-active moiety, the nucleic acid oligomer, or the conductive surface, for example via the solution added to the modified conductive surface, the substrates specific to the catalytically redox-active moiety, in particular, being able to act as external oxidizing or reducing agents. In addition, an external oxidizing or reducing agent can also be covalently joined with the nucleic acid oligomer, the oxidizing or reducing agent being covalently attached to the nucleic acid oligomer at a site that is situated at least two covalently-joined nucleotides or at least two covalently-joined pyrimidine or purine bases away from the covalent attachment site of the catalytically redox-active moiety, preferably at the oligonucleotide end opposite the modification with the redox-active moiety, near the conductive surface. In particular, also the conductive surface (electrode) may act as the external oxidizing or reducing agent. The ability to act as an electron acceptor or donor is relative, i.e. a molecule that acts as an electron acceptor toward another molecule, directly or under the influence of certain external conditions, may also act as an electron donor toward that molecule under differing experimental conditions, or toward a third molecule under the same or differing experimental conditions.

oxidizing agent — A chemical compound (chemical substance) that oxidizes another

	the electrode also may represent the reducing agent.
redox-active	Redox-active refers to the property of a redox-active moiety of giving up electrons to a suitable oxidizing agent or taking up electrons from a suitable reducing agent under certain external conditions, or the property of a redox-active substance of giving up electrons to a suitable electron acceptor or taking up electrons from a suitable electron donor under certain external conditions.
analyte	Equivalent to a substrate.
substrate	A free oxidizing or reducing agent not covalently joined with but in contact with the catalytically redox-active moiety, the nucleic acid oligomer, or the conductive surface, for example via the solution applied to the modified conductive surface, the substrate being able to be for example a charged or uncharged molecule, any salt, an ion, or a redox-active protein or enzyme (oxido-reductase). The substrate is characterized in that it is recognized by the catalytically redox-active moiety due to the formation of specific interactions between the substrate and the catalytically redox-active moiety and can reduce the donor (or oxidize the acceptor) of the catalytically redox-active moiety, the catalytic activity of the catalytically redox-active moiety accelerating (catalyzing) this redox reaction of the substrate to the product.
catalytic activity	The catalytic activity of the catalytically redox-active moiety has an accelerating effect on the specific reaction between the moiety and the affiliated substrate and thus allows a reaction course in which the catalytic activity of the moiety is imperceptible or nonexistent. This catalytic activity of the redox-active moiety is achieved by stabilizing the relevant transitional state, i.e. the highest-energy species, in the reaction course between the catalytically redox-active moiety and the affiliated substrate.
electrocatalytic activity	The electrocatalytic activity of the catalytically redox-active moiety is

chemical compound (chemical substance, electron donor, electron acceptor) by taking up electrons from this other chemical compound (chemical substance, electron donor, electron acceptor). An oxidizing agent behaves analogously to an electron acceptor, but is used in the context of the present invention to denote an external electron acceptor not directly belonging to the catalytically redox-active moiety. In this context, "not directly" means that the oxidizing agent is either a substrate specific to the catalytically redox-active moiety, or a free redox-active substance that is not bound to but in contact with the nucleic acid oligomer. In addition, the oxidizing agent may be covalently attached to the nucleic acid oligomer, but at a nucleic acid oligomer site that is situated at least two covalently-joined nucleotides or at least two covalently-joined pyrimidine or purine bases away from the covalent attachment site of the catalytically redox-active moiety. In particular, the electrode also may represent the oxidizing agent.

reducing agent

A chemical compound (chemical substance) that, by giving up electrons to another chemical compound (chemical substance, electron donor, electron acceptor), reduces this other chemical compound (chemical substance, electron donor, electron acceptor). A reducing agent behaves analogously to an electron donor but is used in the context of the present invention to denote an external electron donor not directly belonging to the catalytically redox-active moiety. In this context, "not directly" means that the reducing agent is either a substrate specific to the catalytically redox-active moiety, or a free redox-active substance that is not bound to but in contact with the nucleic acid oligomer, or that the reducing agent is covalently attached to the nucleic acid oligomer, but at a nucleic acid oligomer site that is situated at least two covalently-joined nucleotides or at least two covalently-joined pyrimidine or purine bases away from the covalent attachment site of the redox-active moiety. In particular,

electron acceptor, directly or under the influence of certain external conditions. For example, one such external condition is the oxidation or reduction of the electron donor or acceptor of the catalytically redox-active moiety by an external oxidizing or reducing agent, so for example the transfer of an electron to the electron donor by a reducing agent, or the release of an electron by the electron acceptor to an oxidizing agent. These oxidizing or reducing agents may be external redox-active substances, i.e. they are not covalently joined with but in contact with the catalytically redox-active moiety, the nucleic acid oligomer, or the conductive surface, for example via the solution added to the modified conductive surface, the substrates specific to the catalytically redox-active moiety, in particular, being able to act as external oxidizing or reducing agents. In addition, an external oxidizing or reducing agent also may be covalently joined with the nucleic acid oligomer, the oxidizing or reducing agent being covalently attached to the nucleic acid oligomer at a site that is situated at least two covalently-joined nucleotides or at least two covalently-joined pyrimidine or purine bases away from the covalent attachment site of the redox-active moiety, preferably at the oligonucleotide end opposite the modification with the catalytically redox-active moiety, near the conductive surface. In particular, also the conductive surface (electrode) may act as the external oxidizing or reducing agent. The ability to act as an electron donor or acceptor is relative, i.e. a molecule that acts as an electron donor toward another molecule, directly or under the influence of certain external conditions, may also act as an electron acceptor toward that molecule under differing experimental conditions, or toward a third molecule under the same or differing experimental conditions.

electron acceptor

In the context of the present invention, the term "electron acceptor" refers to a component of a catalytically redox-active moiety. An

	macromolecules binding these redox-active centers.
cofactor	Equivalent to a redox-active center (electron donor or acceptor) of the catalytically redox-active moiety.
prosthetic group	Equivalent to a redox-active center (electron donor or acceptor) of the catalytically redox-active moiety.
redox-active center of the catalytically redox-active moiety	The redox-active center of the catalytically redox-active moiety is characterized in that it acts as an electron donor or acceptor toward a substrate specific to the catalytically redox-active moiety. Moreover, if a catalytically redox-active moiety possesses multiple redox-active centers (electron donors and/or electron acceptors), a charge transfer may occur within the catalytically redox-active moiety: following the charge transfer between the substrate specific to the catalytically redox-active moiety and a first redox-active center, an additional charge transfer is possible between this first redox-active center and an additional redox-active center of the same catalytically redox-active moiety, this second redox-active center, in turn, being able to transfer charge to a third redox-active center, and so on. Thus, a successive charge transfer may occur within the catalytically redox-active moiety if the catalytically redox-active moiety includes multiple redox-active centers. In this case, the process of successive charge transfer is initiated by the presence of the substrate (with its property of spontaneously transferring a charge between the substrate and the catalytically redox-active moiety) specific to the catalytically redox-active moiety.
electron-donor molecule	Equivalent to an electron donor.
electron-acceptor molecule	Equivalent to an electron acceptor.
electron donor	In the context of the present invention, the term "electron donor" refers to a component of the catalytically redox-active moiety. An electron donor is a molecule that can transfer an electron to an

closely related to the catalytic activity of the moiety. The presence of the catalytically redox-active moiety and its integration in the course of the electrode reaction of the substrate to the product (the course of the entire electrochemical redox reaction between an electrode and the substrate, i.e. the electrode giving up electrons to the substrate or the substrate giving up electrons to the electrode, to the intermediate steps of the redox reaction between the substrate and the catalytically redox-active moiety and the redox reaction between the redox-active moiety and the electrode) accelerates the electrochemical conversion of the substrate at the electrode. The electrocatalytic activity of a catalytically redox-active moiety immobilized at an electrode reduces the activation energy of the electrode reaction of the substrate to the product (the energy of the highest-energy state for the course of the conversion of the substrate to the product at the electrode) and thus causes a shift in the electrode potential required for the electrode reaction of the substrate to the product, in the direction of the equilibrium potential for this electrode reaction. Decreasing the activation potential causes a reduction of the overpotential required for an electrode reaction, and thus an increase in the flow of electrons between the electrode and the substrate at a specific electrode potential that is suitable for the electrode reaction (this increase is generally referred to as "catalytic current"). An important result of the electrocatalytic activity is thus that the electrochemical conversion of the substrate to the product can be carried out in the presence and with the participation of the catalytically redox-active moiety at an electrode potential at which, in the absence of the catalytically redox-active moiety, very little or no current flows.

specificity of the catalytically redox-active moiety

The catalytically redox-active moiety acts specifically both with a view to the substrate that interacts with the catalytically redox-active moiety and with a view to the reaction carried out with the relevant

substrate. In the context of the present invention, redox reactions are the preferred reactions between the catalytically redox-active moiety and the substrate.

initiation process

Given appropriately chosen external conditions, the catalytically redox-active moiety exhibits its redox activity, in other words its property of for example giving up electrons to a suitable oxidizing agent or taking up electrons from a suitable reducing agent, only subsequent to an initiation process. Such an initiation process may be the addition of substrate with its property of transferring charge to the catalytically redox-active moiety: thus, the reductive property of a catalytically redox-active moiety becomes possible only through the transfer of the electron(s) from the substrate to the/an electron donor "D," either in the presence of an oxidizing agent that can oxidize D^- but not D, or because, subsequent to successive charge transfer within the catalytically redox-active moiety, the electron is transferred from D^- to an acceptor "A" (directly or via multiple electron transfer steps to intermediate electron acceptors) and an oxidizing agent is present that takes up electrons only from this reduced acceptor "A^-" of the catalytically redox-active moiety, but not from A. In particular, this oxidizing agent may also be an electrode, for example if the electrode is set to a potential at which D^- but not D (or A^- but not A) is oxidized. On the other hand, the oxidative property of a catalytically redox-active moiety becomes possible only through the transfer of the electron(s) from an electron donor "D" to the substrate, either in the presence of a reducing agent that can reduce D^+ but not D, or because, subsequent to successive charge transfer within the catalytically redox-active moiety, an electron is transferred from an acceptor "A" to the oxidized donor D^+ (directly or via multiple electron transfer steps from intermediate electron donors) and a reducing agent is present that gives up electrons only to this oxidized acceptor "A^+"

	of the catalytically redox-active moiety, but not to A. In particular, this reducing agent may also be an electrode, for example if the electrode is set to a potential at which D^+ but not D (or A^+ but not A) is reduced.
redox-active protein/enzyme	Usually consists of what is known as 'apoprotein,' the preferred macromolecule(s) of the present invention, and cofactors, the electron donor(s) and/or electron acceptor(s) within the meaning of the present invention. The redox activity of the redox-active protein/enzyme is triggered by a free redox-active substance (the specific substrate).
oxidase	A class of redox-active enzymes that catalyze the oxidation of the substrate specific to the relevant oxidase.
reductase	A class of redox-active enzymes that catalyze the reduction of the substrate specific to the relevant reductase.
oxido-reductases	Generic term for oxidases and reductases.
GOx	Glucose oxidase (β-D-glucose: oxygen 1-oxido-reductase, EC 1.1.3.4). An example of a redox-active protein/enzyme. The protein/enzyme is an enzyme comprising apoprotein and FAD as a cofactor, cf. Structure 1 and Formula 1. The GOx is present as a homodimeric enzyme (Hecht et al., J. Mol. Biol. 229 (1993), pp. 153-172).
ADH	Alcohol dehydrogenase (EC 1.1.1.1). An example of a redox-active protein/enzyme. The protein/enzyme is an enzyme comprising apoprotein comprising three protein subunits and PQQ, heme, and a heme dimer as cofactors (Amayama et al., Methods Enzymol. 89 (1982) 450-457).
FDH	Fructose dehydrogenase (EC 1.1.99.11). An example of a redox-active protein/enzyme. The protein/enzyme is an enzyme comprising apoprotein and PQQ as (one of) the cofactor(s). The structure of this enzyme is unknown.
LDH	Lactate dehydrogenase (EC 1.1.1.27), an enzyme comprising apoprotein, FMN, and heme.
FAD	flavin adenine dinucleotide, cf. Formula 1

NAD^+	nicotinamide adenine dinucleotide, cf. Formula 2
PQQ	Pyrroloquinoline quinone; corresponds to 4,5-dihydro-4,5-dioxo-1H-pyrrolo-[2,3-f]-quinoline-2,7,9-tricarboxylic acid, cf. Formula 3 ($R_1 = R_3 = R_5 = CO_2H$; $R_2 = R_4 = H$) or a derivative thereof (Formula 3).
Heme	Iron-protoporphyrin IX, Formula 4 with $R_2 = R_5 = R_8 = R_{10} = H$; $R_4 = R_6 = R_9 = R_{12} = CH_3$; $R_1 = R_3 = CH_2\text{-}CH_2\text{-}CO_2^-$; $R_7 = R_9 = CH{=}CH_2$, or a derivative of iron-protoporphyrin (Formula 4).
N^6-(2-aminoethyl)-FAD	modified flavin adenine dinucleotide, cf. Formula 5
N^6-(2-aminoethyl)-NAD^+	modified nicotinamide adenine dinucleotide, cf. Formula 6
EDTA	ethylenediamine tetraacetate (sodium salt)
sulfo-NHS	N-hydroxysulfosuccinimide
EDC	(3-dimethylaminopropyl)-carbodiimide
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Tris	trishydroxymethylamino methane
alkyl	The term "alkyl" refers to a saturated hydrocarbon group that is straight-chain or branched (e.g. ethyl, 2,5-dimethylhexyl, or isopropyl, etc.). When "alkyl" is used to indicate a linker or spacer, the term refers to a group having two available valences for covalent linkage (e.g. $-CH_2CH_2-$, $-CH_2C(CH_3)_2CH_2CH_2C(CH_3)_2CH_2-$ or $-CH_2CH_2CH_2-$, etc.). Alkyl groups preferred as substituents or side chains R are those having a chain length of 1 - 30 (the longest continuous chain of atoms covalently bound to one another). Alkyl groups preferred as linkers or spacers are those having a chain length of 1 - 20, especially a chain length of 1 - 14, the chain length here representing the shortest continuous link between the structures joined via the linker or spacer, in other words between the two molecules or between a surface atom, surface molecule, or surface molecule group and another molecule.
alkenyl	Alkyl groups in which one or more of the C-C single bonds are

	replaced by C=C double bonds.
alkynyl	Alkyl or alkenyl groups in which one or more of the C-C single or C=C double bonds are replaced by C≡C triple bonds.
heteroalkyl	Alkyl groups in which one or more of the C-H bonds or C-C single bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
heteroalkenyl	Alkenyl groups in which one or more C-H bonds, C-C single or C=C double bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
heteroalkynyl	Alkynyl groups in which one or more of the C-H bonds, C-C single, C=C double or C≡C triple bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
linker	A molecular link between two molecules or between a surface atom, surface molecule, or surface molecule group and another molecule. Linkers can usually be purchased in the form of alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, or heteroalkynyl chains, the chain being derivatized in two places with (identical or different) reactive groups. These groups form a covalent chemical bond in simple/known chemical reactions with the appropriate reaction partners. The reactive groups may also be photoactivatable, i.e. the reactive groups are activated only by light of a specific or any given wavelength. Preferred linkers are those having a chain length of 1 - 20, especially a chain length of 1 - 14, the chain length here representing the shortest continuous link between the structures to be joined, in other words between the two molecules or between a surface atom, surface molecule, or surface molecule group and another molecule.
spacer	A linker that is covalently attached via the reactive groups to one or both of the structures to be joined (see linker). Preferred spacers are those having a chain length of 1 - 20, especially a chain length of 1 - 14, the chain length representing the shortest continuous link between the structures to be joined.

(n x HS-spacer)-oligo	A nucleic acid oligomer to which n thiol functions are each attached via a spacer, each spacer being able to have a different chain length (the shortest continuous link between the thiol function and the nucleic acid oligomer), especially any chain length between 1 and 14. These spacers, in turn, may be bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been affixed thereto by modification, and "n" is any integer, especially a number between 1 and 20.
(n x R-S-S-spacer)-oligo	A nucleic acid oligomer to which n disulfide functions are each attached via a spacer, the disulfide function being saturated by any residue R. Each spacer for attaching the disulfide function to the nucleic acid oligomer may have a different chain length (shortest continuous link between the disulfide function and the nucleic acid oligomer), especially any chain length between 1 and 14. These spacers, in turn, may be bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been affixed thereto by modification. The variable n is any integer, especially a number between 1 and 20.
oligo-spacer-S-S-spacer-oligo	Two identical or different nucleic acid oligomers that are joined with each other via a disulfide bridge, the disulfide bridge being attached to the nucleic acid oligomers via any two spacers, the two spacers being able to have differing chain lengths (the shortest continuous link between the disulfide bridge and the respective nucleic acid oligomer), especially any chain length between 1 and 14, and these spacers, in turn, being able to be bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been affixed thereto by modification.
mica	Muscovite lamina, a support material for the application of thin films.
Au-S-$(CH_2)_2$-ss-oligo-spacer-PQQ-FAD(GOx)	Gold film on mica having a covalently applied monolayer comprising derivatized 12-bp single-strand DNA oligonucleotide (sequence: TAGTCGGAAGCA). Here, the oligonucleotide's terminal phosphate group at the 3'-end is esterified with $(HO\text{-}(CH_2)_2\text{-}S)_2$ to form P-O-

	$(CH_2)_2$-S-S-$(CH_2)_2$-OH, the S-S bond being homolytically cleaved and producing one Au-S-R bond each. The terminal thymine base at the 5'-end of the oligonucleotide is modified at the C-5 carbon with -CH=CH-CO-NH-CH_2-CH_2-NH_2, this residue, in turn, being joined via its free amino group with the carboxylic-acid group of the PQQ by amidation. To a further carboxylic-acid group of this PQQ is bound by amidation FAD that was previously modified in such a way that it has a reactive amino group at its disposal, for example by forming N^6-(2-aminoethyl)-FAD (Bückmann et al., 1991, European Patent 0.247.537.B1). Thereafter, the FAD is reconstituted with the apoprotein of the GOx such that a nucleic acid oligomer results that is covalently attached to the surface and, in addition – via PQQ as a covalently attached bridge molecule –, is covalently modified with the complete GOx moiety.
Au-S-$(CH_2)_2$-ds-oligo-spacer-PQQ-FAD(GOx)	*Au-S-$(CH_2)_2$-ss-oligo-spacer-PQQ-FAD(GOx)* hybridized with the oligonucleotide that is complementary to the ss-oligo (sequence: TAGTCGGAAGCA).
Au-S-$(CH_2)_2$-ss-oligo-spacer-PQQ-NAD^+-LDH	Gold film on mica having a covalently applied monolayer comprising derivatized 12-bp single-strand DNA oligonucleotide (sequence: TAGTCGGAAGCA). Here, the oligonucleotide's terminal phosphate group at the 3'-end is esterified with (HO-$(CH_2)_2$-$S)_2$ to form P-O-$(CH_2)_2$-S-S-$(CH_2)_2$-OH, the S-S bond being homolytically cleaved and producing one Au-S-R bond each. The terminal thymine base at the 5'-end of the oligonucleotide is modified at the C-5 carbon with -CH=CH-CO-NH-CH_2-CH_2-NH_2, this residue, in turn, being joined via its free amino group with the carboxylic-acid group of the PQQ by amidation. To a further carboxylic-acid group of this PQQ is bound by amidation NAD^+ that was previously modified in such a way that it has a reactive amino group at its disposal, for example by forming N^6-(2-aminoethyl)-NAD^+ (Bückmann et al., 1991, European Patent 0.247.537.B1). The complete LDH is associated at this terminal NAD^+.
Au-S-$(CH_2)_2$-ds-oligo-spacer-PQQ-NAD^+-	*Au-S-$(CH_2)_2$-ss-oligo-spacer-PQQ-NAD^+-ADH* hybridized with the oligonucleotide that is complementary to the ss-oligo (sequence:

LDH	TAGTCGGAAGCA).
E	The electrode potential on the working electrode.
E^{eq}_{0}	The equilibrium potential of an electrode reaction.
	The "zero current" potential of an electrode reaction, the potential that does not supply total current (the sum of the oxidative and reductive current) for a specific electrode reaction.
h	The overpotential of an electrode reaction.
Electrode reaction	The redox reaction between a redox-active substance and an electrode (taking up electrons from the electrode by the redox-active substance or giving up electrons from the redox-active substance to the electrode).
E_{Ox}	The potential at maximum current of the oxidation of a reversible electrooxidation or electroreduction.
E_{Red}	The potential at maximum current of the reduction of a reversible electrooxidation or electroreduction.
I	current density (current per cm^2 of electrode surface)
cyclic voltammetry	Recording a current-voltage curve. Here, the potential of a stationary working electrode is changed linearly as a function of time, starting at a potential at which no electrooxidation or electroreduction occurs, up to a potential at which a species that is dissolved or adsorbed on the electrode is oxidized or reduced (i.e. a current flows). After running through the oxidation or reduction operation, which produces in the current-voltage curve an initially increasing current and, after reaching a maximum, a gradually decreasing current, the direction of the potential feed is reversed. The behavior of the products of electrooxidation or electroreduction is then recorded in a reverse run.

amperometry	Recording a current-time curve. Here, the potential of a stationary working electrode is set, for example by a potential jump, to a potential at which the electrooxidation or electroreduction of a dissolved or adsorbed species occurs, and the flowing current is recorded as a function of time.
potentiometry	Recording an electrode voltage course as a function of, for example, substrate consumption. Here, the potential of a stationary working electrode is set, for example, to the "zero current" potential E^0 of the substrate. When the substrate is consumed by the catalytically redox-active moiety (in the case of hybridization), the "zero current" potential E^0 changes in the direction of the equilibrium potential E^{eq}. Thus, recording the potential as a function of time (~ substrate consumption) provides information on the hybridization state.

[018] The present invention is directed to a nucleic acid oligomer that is modified by chemically binding a catalytically redox-active moiety. After giving up an electron to an external oxidizing agent (substrate), the catalytically redox-active moiety may be reduced by an external reducing agent, for example an electrode, or after taking up an electron from an external reducing agent (substrate), be oxidized by an external oxidizing agent, for example an electrode.

[019] In the context of the present invention, a compound comprising at least two covalently-joined nucleotides or at least two covalently-joined pyrimidine (e.g. cytosine, thymine, or uracil) or purine bases (e.g. adenine or guanine), preferably a DNA, RNA, or PNA fragment, is used as the nucleic acid oligomer. In the present invention, the term "nucleic acid" refers to any backbone of the covalently-joined pyrimidine or purine bases, such as the sugar-phosphate backbone of DNA, cDNA, or RNA, a peptide backbone of PNA, or analogous backbone structures such as a thiophosphate, a dithiophosphate, or a phosphoramide backbone. An essential feature of a nucleic acid within the meaning of the present invention is that it can sequence-specifically bind naturally occurring DNA or RNA. The terms "(probe) oligonucleotide", "nucleic acid", and "oligomer" are used as alternatives to the term "nucleic acid oligomer".

[020] In the context of the present invention, the term "electron acceptor" or "electron-acceptor molecule" and the term "electron donor" or "electron-donor molecule" refer to a component (a redox-active center, cofactor, or prosthetic

group) of a catalytically redox-active moiety.

[021] In the context of the present invention, a moiety referred to using the generic term "catalytically redox-active moiety" usually consists of one or more redox-active centers (cofactors, prosthetic groups), referred to in the following as electron donors or electron acceptors, and one or more macromolecules binding these redox-active centers. Thus, in its form that is relevant to the present invention, the catalytically redox-active moiety contains one or more electron-donor molecules and/or one or more electron-acceptor molecules, this (these) electron-donor molecule(s) and/or this (these) electron-acceptor molecule(s) being bound to one or more macromolecules or being embedded in this (these) macromolecule(s). Electron donor(s) and/or electron acceptor(s) may be joined with one another via one or more covalent or ionic bonds, via hydrogen bonds, van der Waals bridges, via π-π-interaction, or via coordination by means of electron-pair donation and acceptance, the covalent links being able to be direct or indirect links (e.g. via a spacer, but not via a nucleic acid oligomer). In addition, the electron donor(s) and/or electron acceptor(s) may be joined with the macromolecule(s) via covalent attachment to the macromolecule(s), via encapsulation in suitable molecular cavities (binding pockets) of the macromolecule(s), via ionic bonds, hydrogen bonds, van der Waals bridges, π-π-interaction, or via coordination by means of electron-pair donation and acceptance between the macromolecule(s) and the electron-donor molecule(s) and/or the electron-acceptor molecule(s). If multiple macromolecules are components of the catalytically redox-active moiety, the binding of the macromolecules to one another may likewise take place covalently, ionically, via hydrogen bonds, van der Waals bridges, π-π-interaction, or via coordination by means of electron-pair donation and acceptance. In the minimum case, a catalytically redox-active moiety may also consist of only one macromolecule, the macromolecule, in its form that is relevant to the present invention, then also acting as the electron donor or acceptor. It may also consist of only one electron donor or acceptor. In addition, the catalytically redox-active moiety may also be formed through spontaneous congregation of the components in solution (in situ).

[022] The aforementioned donor and/or acceptor molecules form, together with the macromolecules, a catalytically redox-active moiety, i.e., they are bound to one another directly or via further molecular moieties. The sole restriction on the

molecules or molecular moieties joining the components of the catalytically redox-active moiety is the exclusion of nucleic acid oligomers. According to the present invention, the catalytically redox-active moiety is bound to the probe oligonucleotide as a complete moiety, multiple chemical bonds being able, of course, to be formed between the oligonucleotide and the redox-active moiety. The exclusion of nucleic acid oligomers as the molecules or molecular moieties joining the components of the catalytically redox-active moiety is intended to demonstrate clearly that it is not individual portions of the catalytically redox-active moiety that are attached at various sites of the probe oligonucleotide. Thus, the probe oligonucleotide explicitly does not represent the link between the electron-donor molecule(s) and the macromolecules and/or the electron-acceptor molecule(s) and the macromolecules of the catalytically redox-active moiety.

[023] The redox activity of the catalytically redox-active moiety, in other words its property of giving up electrons to a suitable oxidizing agent under certain external conditions (or taking up electrons from a suitable reducing agent), is exhibited through an initiation process, for example only subsequent to reduction (or subsequent to oxidation) by the substrate. Given appropriately chosen external conditions, the catalytically redox-active moiety exhibits its redox activity only subsequent to the initiation process "adding substrate having the property of transferring charge to the catalytically redox-active moiety": thus, the reductive property of a catalytically redox-active moiety becomes possible only through the transfer of the electron(s) from the substrate to the/an electron donor "D," either in the presence of an external oxidizing agent (e.g. the electrode having an appropriately chosen potential) that can oxidize D^- but not D, or because, subsequent to successive charge transfer within the catalytically redox-active moiety, the electron is transferred from D^- to an acceptor "A" (directly or via multiple electron transfer steps to intermediate electron acceptors) and an oxidizing agent is present that takes up electrons from only this reduced acceptor "A^-" of the catalytically redox-active moiety, but not from A (e.g. in the presence of an electrode having an appropriately chosen potential). On the other hand, the oxidative property of a catalytically redox-active moiety becomes possible only through the transfer of the electron(s) from an electron donor "D" to the substrate, either in the presence of a reducing agent (e.g. the electrode having an appropriately chosen potential) that can reduce D^+ but not D, or because,

subsequent to successive charge transfer within the catalytically redox-active moiety, an electron is transferred from an acceptor "A" to the oxidized donor D^+ (directly or via multiple electron transfer steps from intermediate electron donors) and a reducing agent is present that gives up electrons only to this oxidized acceptor "A^{+}" of the catalytically redox-active moiety but not to A (e.g. in the presence of an electrode having an appropriately chosen potential).

[024] In addition to comprising electron donor(s) and/or electron acceptor(s) and macromolecule(s), essential features of the catalytically redox-active moiety are: (i) in the forms relevant to the present invention (electron donor(s) and/or electron acceptor(s and macromolecule(s)) in their original state or in an oxidized or reduced state), the moiety is stable and does not dissociate into its components, (ii) the electrocatalytic activity of the moiety (see below), (iii) the moiety includes no nucleic acid, (iv) the moiety's composition comprising electron donor(s) and/or electron acceptor(s) and macromolecule(s) can be recognized by a person skilled in the art, regardless of the bond between the components, since, in principle, the redox-active centers (cofactors, prosthetic groups) and the affiliated matrix comprising macromolecule(s) (e.g. the apoprotein in the case of enzymes, as an example of a catalytically redox-active moiety) may also occur separately.

[025] The substrate specific to a particular catalytically redox-active moiety is a free oxidizing or reducing agent not covalently joined with but in contact with the catalytically redox-active moiety, the nucleic acid oligomer, or the conductive surface, for example via the solution applied to the modified conductive surface, the substrate being able to be for example a charged or uncharged molecule, any salt, an ion, or a redox-active protein or enzyme (oxido-reductase). The substrate is characterized in that it is recognized by the catalytically redox-active moiety due to the formation of specific interactions between the substrate and the catalytically redox-active moiety and can reduce the donor (or oxidize the acceptor) of the catalytically redox-active moiety, the catalytic activity of the catalytically redox-active moiety accelerating (catalyzing) this redox reaction of the substrate to the product.

[026] The catalytic activity of the catalytically redox-active moiety has an accelerating effect on the specific reaction between the moiety and the affiliated substrate and thus allows a reaction course in which the catalytic activity of the moiety (e.g. in the form of

the substrate and the non-bound cofactor in solution) is imperceptible or nonexistent. This catalytic activity of the redox-active moiety is achieved by stabilizing the relevant transitional state, i.e. the highest-energy species, in the course of the reaction between the catalytically redox-active moiety and the affiliated substrate.

[027] The electrocatalytic activity of the catalytically redox-active moiety is closely related to the catalytic activity of the moiety. The presence of the catalytically redox-active moiety and its integration in the course of the electrode reaction of the substrate to the product (the entire course of the electrochemical redox reaction between an electrode and the substrate, i.e. the electrode giving up electrons to the substrate or the substrate giving up electrons to the electrode, to the intermediate steps of the redox reaction between the substrate and the catalytically redox-active moiety and the redox reaction between the redox-active moiety and the electrode) accelerates the electrochemical conversion of the substrate at the electrode. The electrocatalytic activity of a catalytically redox-active moiety immobilized at an electrode reduces the activation energy of the electrode reaction of the substrate to the product (the energy of the highest-energy state for the course of the conversion of the substrate to the product at the electrode) and thus causes a shift in the electrode potential required for the electrode reaction of the substrate to the product, in the direction of the equilibrium potential for this electrode reaction. Decreasing the activation potential causes a reduction of the overpotential required for an electrode reaction, and thus an increase in the flow of electrons between the electrode and the substrate at a particular electrode potential that is suitable for the electrode reaction (this increase is generally referred to as "catalytic current"). An important result of the electrocatalytic activity is thus that the electrochemical conversion of the substrate to the product can be carried out in the presence and with the participation of the catalytically redox-active moiety at an electrode potential at which, in the absence of the catalytically redox-active moiety, very little or no current flows.

[028] The catalytically redox-active moiety acts specifically both with a view to the substrate that interacts with the catalytically redox-active moiety and with a view to the reaction carried out with the relevant substrate. In the context of the present invention, redox reactions are the preferred reactions between the catalytically redox-active moiety and the substrate.

[029] In the context of the present invention, the term "reducing agent" refers to a chemical compound (chemical substance) that, by giving up electrons to another chemical compound (chemical substance, electron donor, electron acceptor), reduces this other chemical compound (chemical substance, electron donor, electron acceptor). The reducing agent behaves analogously to an electron donor but is used in the context of the present invention to denote an external electron donor not directly belonging to the redox-active moiety. In this context, "not directly" means that the reducing agent is either a free redox-active substance that is not bound to but in contact with the nucleic acid oligomer, or that the reducing agent is covalently attached to the nucleic acid oligomer, but at a nucleic acid oligomer site that is situated at least two covalently-joined nucleotides or at least two covalently-joined pyrimidine or purine bases away from the covalent attachment site of the redox-active moiety. In particular, the electrode may represent the reducing agent.

[030] In the context of the present invention, the term "free redox-active substance" refers to a free oxidizing or reducing agent not covalently joined with but in contact with the redox-active moiety, the nucleic acid oligomer, or the conductive surface, for example via the solution applied to the modified conductive surface, the free redox-active substance being able to be for example an uncharged molecule, any salt, an ion, or a redox-active protein or enzyme (oxido-reductase). The free redox-active substance is characterized in that it can reduce the donor (or oxidize the acceptor) of the catalytically redox-active moiety. In particular, the specific substrate of the catalytically redox-active moiety is a free redox-active substance.

[031] The modified nucleic acid oligomer is directly or indirectly (via a spacer) bound to a conductive surface. The term "conductive surface" is understood to mean any electrically conductive surface of any thickness, especially metallic surfaces, surfaces comprising metal alloys, or doped or non-doped semiconductor surfaces, all semiconductors being able to be used in the form of pure substances or in the form of mixtures. In the context of the present invention, the conductive surface may be present alone or applied to any support material such as glass. In the context of the present invention, the term "electrode" is used as an alternative to "conductive surface".

[032] The term "modified conductive surface" is understood to mean a conductive surface that is modified by attaching a nucleic acid oligomer modified with a catalytically redox-active moiety. In the context of the present invention, the term "functionalized electrode" is used as an alternative to the term "modified conductive surface".

[033] According to a further aspect, the present invention is directed to a method that allows the electrochemical detection of molecular structures, such as the detection of the substrate, but in particular the electrochemical detection of DNA/RNA/PNA fragments in a probe solution by sequence-specific nucleic acid oligomer hybridization. The detection of the hybridization events via electrical signals is a simple and economical method and, in a battery-operated variation, allows on-site application.

[034] The present invention further provides a read-out method for detecting molecular structures, inter alia for parallel detection of hybridization events on an oligomer chip by reading out electrical signals within a microelectrode array. According to the present invention, a "microelectrode-targetable read-out method" is understood to be a method in which the detection of molecular structures on a specific electrode within the electrode array functionalized with catalytically redox-active moieties is achieved by electrically targeting this electrode, for example directly or using cMOS technology. Furthermore, parallel detection of hybridization events may also be achieved either by using various catalytically redox-active moieties for the individual electrodes of the array when forming the various functionalized electrodes of an electrode array or by using a continuously conductive surface to form the functionalized electrodes and by achieving the differentiability of molecular structures on a specific area having an identical electrode structure (to that of a specific test site) within the entire system (of the complete oligomer chip) by using, for the individual test sites, various catalytically redox-active moieties that can be addressed by selectively adding the relevant specific substrate. In the latter variant, the electrochemical response of the entire oligomer chip is detected as a result of the continuous conductive surface; addressing and reading out the electrochemical response of individual test sites

occurs by selectively adding the relevant specific substrate for this test site.

[035] Furthermore, in the embodiment of an electrode array comprising electrodes, each of which was functionalized with a different catalytically redox-active moiety, the invention represents a microelectrode-targetable method of qualitatively and quantitatively detecting, in parallel, redox-active substances, the relevant substrate of the various catalytically redox-active moieties of the electrodes within an electrode array.

Binding a Catalytically Redox-Active Moiety to a Nucleic Acid Oligomer

[036] A prerequisite for the method according to the present invention is the binding of a catalytically redox-active moiety to a nucleic acid oligomer. The catalytically redox-active moiety may be for example any redox-active protein/enzyme from the group of oxidases or reductases, protein-engineering or gene-mutation-modified proteins/enzymes from this group of oxidases or reductases, or an artificially produced moiety comprising one or more redox-active centers (electron donor or acceptor), or an artificially produced moiety comprising one or more redox-active centers (electron donor or acceptor) and one or more macromolecules binding these redox-active centers.

[037] The following are some examples of a catalytically redox-active moiety:

(i) Redox-active proteins/enzymes, such as the oxido-reductases, some of which are compiled in Table 1 below. The covalent attachment of the catalytically redox-active moiety (the redox-active protein/enzyme) occurs, in the context of the present invention, preferably via a covalent attachment of the cofactor with subsequent reconstitution of the apoprotein to the cofactor attached to the nucleic acid oligomer. In catalytically redox-active moieties (redox-active proteins/enzymes) having multiple cofactors, one of the cofactors (appears in bold in Table 1 below) is covalently attached to the nucleic acid oligomer, and the catalytically redox-active moiety is completed by reconstituting it with the remaining cofactors and the apoprotein.

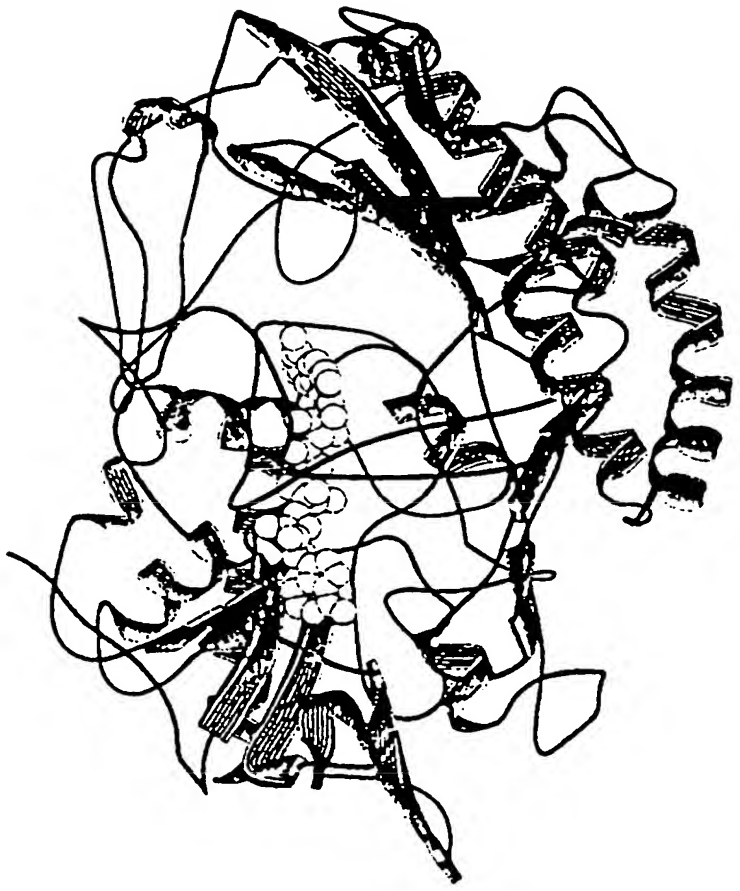
Table 1: A selection of some redox-active enyzmes (oxido-reductases) as examples of catalytically redox-active moieties.

Enzyme	Cofactor	Substrate	Catalyzed enzyme reaction
Glucose oxidase	FAD	Glucose	Glucose + FAD → gluconic acid + $FADH_2$
Alcohol dehydrogenase	**PQQ**, heme, heme dimer	Ethanol	Ethanol + PQQ → acetaldehyde + $PQQH_2$
Fructose dehydrogenase	**PQQ**, heme, ...	Fructose	D-fructose + PQQ → 5-keto-d-fructose + $PQQH_2$
Lactate dehydrogenase	**FMN**, heme	Lactate	Lactate + FMN → pyruvate + $FMNH_2$
Peroxidases (e.g. horseradish peroxidase, lactoperoxidase, cytochrom c peroxidase, fungal peroxidase, etc.)	Heme		

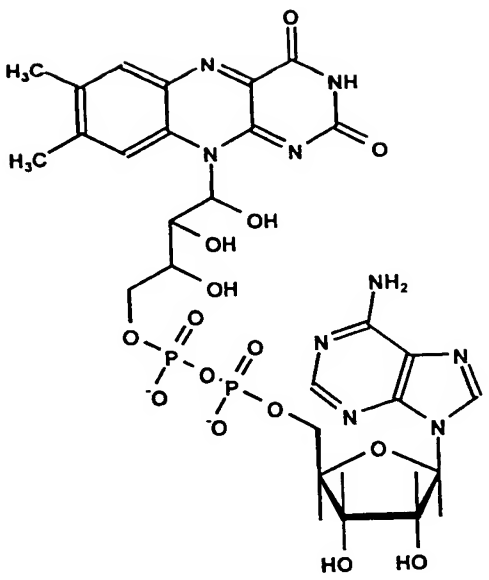
[038] (ii) Modified redox-active proteins/enzymes as described under (i) that were modified through protein engineering or gene mutation and that continue to possess catalytic or electrocatalytic activity.

[039] (iii) Artificially produced catalytically redox-active moieties comprising electron donor(s) and/or electron acceptors and macromolecules possessing catalytic or electrocatalytic activity.

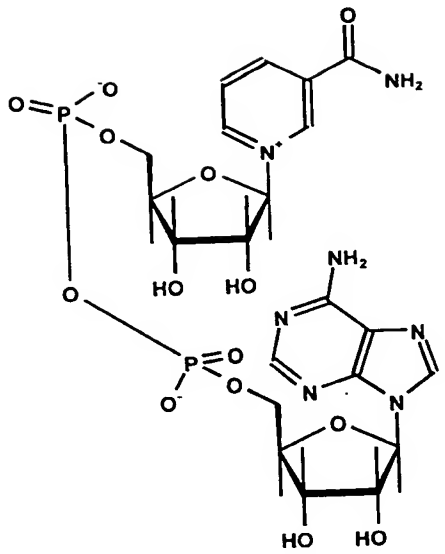
[040] (iv) NAD^+-dependent enzymes such as lactate dehydrogenase (LDH, EC 1.1.1.27) or alcohol dehydrogenase (ADH, EC 1.1.1.1). If NAD^+-dependent enzymes are used, the catalytically redox-active moiety (e.g. LDH or ADH) may be attached to the nucleic acid oligomer by covalently binding (modified) NAD^+ to the nucleic acid oligomer, directly or via a spacer (Example 3), and then associating the NAD^+-dependent enzyme with the (modified) NAD^+ through non-covalent interaction.



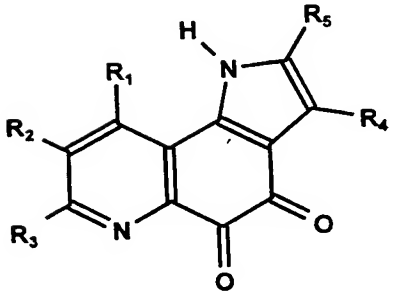
[041] *Structure 1*: Monomer of glucose oxidase (GOx). The apoprotein consists of α-helical and β-pleated sheet domains; the coenzyme flavin adenine dinucleotide (FAD) is drawn in the form of the space-filling shallot model. The structure of the FAD is shown in Formula 1. In its native form, the GOx is present as a homodimer.



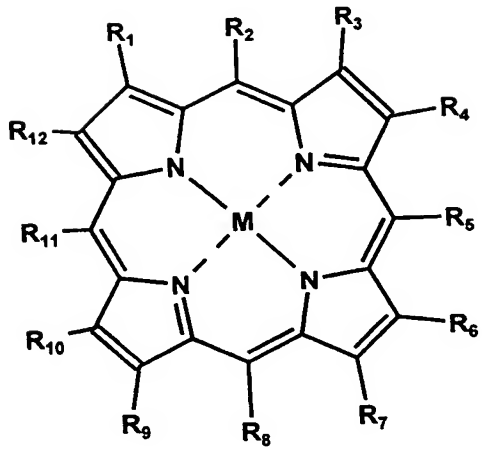
Formula 1



Formula 2

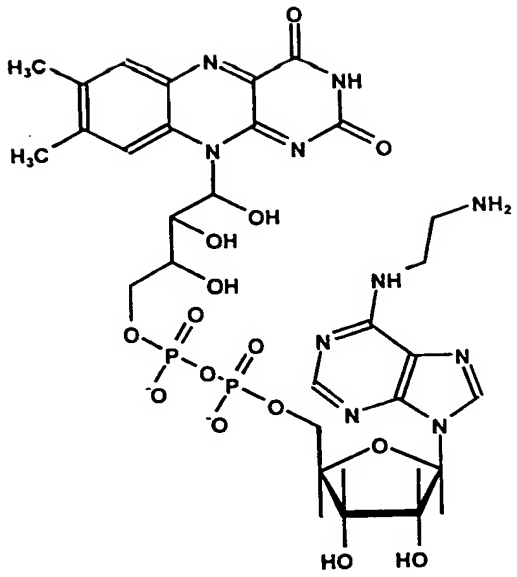


Formula 3

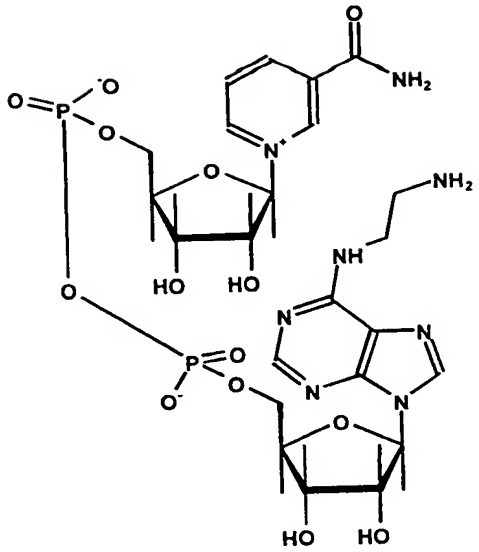


Formula 4

M = 2H, Mg, Zn, Cu, Ni, Pd, Co, Cd, Mn, Fe(II), Fe(III), Sn, Pt, etc.; R_1 to R_{12} are, independently of one another, H or any alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, or heteroalkynyl substituents.



Formula 5



Formula 6

[043] In addition, according to the present invention, a distinguishing feature of the catalytically redox-active moiety is that said moiety gives up electrons to an oxidizing agent that is likewise covalently attached to the nucleic acid oligomer, or takes up electrons from another reducing agent that is likewise covalently attached to the oligonucleotide, this oxidizing or reducing agent being able to be in particular an electrically conductive surface (electrode) and the catalytically redox-active moiety, and especially the redox-active center of the moiety, being able be electrooxidized/electroreduced by applying an external voltage to this electrode in its electrochemically accessible potential range.

[044] According to the present invention, a distinguishing feature of the catalytically redox-active moiety is that the redox-active center of the moiety (directly or subsequent to the specific reaction with the substrate) can be oxidized or reduced at an electrode, and the original state of the catalytically redox-active moiety – prior to the oxidation or reduction at the electrode – is restored through the specific reaction of the catalytically redox-active moiety with the affiliated substrate in a specific catalytic reaction. According to the present invention, any catalytically redox-active moiety may be used for this as long as it or the redox-active center of the catalytically redox-active moiety is oxidizable and reducible at a potential φ that

satisfies the condition 2.0 V $\geq \varphi \geq$ - 2.0 V. The potential refers here to the free, unmodified redox-active center of the catalytically redox-active moiety in a suitable solvent, measured against the normal hydrogen electrode. In the context of the present invention, the potential range 1.7 V $\geq \varphi \geq$ - 1.7 V is preferred, the range 1.4 V $\geq \varphi \geq$ - 1.2 V being particularly preferred and the range 0.9 V $\geq \varphi \geq$ - 0.7 V, in which the redox-active centers of the application examples are oxidized (and rereduced), being most particularly preferred.

[045] A further distinguishing feature of the catalytically redox-active moiety according to the present invention is that, by integrating the catalytically redox-active moiety in the electrochemical oxidation or reduction, the substrate specific to the redox-active center of the moiety is electrocatalytically oxidized or reduced at an electrode, i.e. at a potential at which, in the absence of the catalytically redox-active moiety, very little or no current would flow, or an (additional) catalytic current would result.

[046] According to the present invention, a catalytically redox-active moiety is covalently bound to a nucleic acid oligomer by the reaction of the nucleic acid oligomer with the catalytically redox-active moiety or portions thereof (see also the section "Manner of Executing the Invention"). This bond can be achieved in five different ways:

a) A free phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine group of the oligonucleotide backbone, especially a group at one of the two ends of the oligonucleotide backbone, is used as the reactive group for forming a bond at the nucleic acid oligomer. The free, terminal phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine groups exhibit increased reactivity and thus easily undergo typical reactions such as amidation with (primary or secondary) amino groups or with acid groups; esterification with (primary, secondary, or tertiary) alcohols or with acid groups; thioester formation with (primary, secondary, or tertiary) thioalcohols or with acid groups, or condensation of amine and aldehyde with subsequent reduction of the resultant CH=N bond to a CH_2-NH bond. The coupling group (acid, amine, alcohol, thioalcohol, or aldehyde function) required to covalently

attach the catalytically redox-active moiety is either naturally present on the catalytically redox-active moiety or is obtained by chemically modifying the catalytically redox-active moiety. The attachment of the catalytically redox-active moiety may take place completely or in portions of the moiety with subsequent completion of the catalytically redox-active moiety (see below).

b) The nucleic acid oligomer is modified with a reactive group at the oligonucleotide backbone or at a base via a covalently-attached molecular moiety (spacer) of any composition and chain length (longest continuous chain of atoms bound to one another), especially a chain length of 1 to 14. The modification preferably takes place at one of the ends of the oligonucleotide backbone or at a terminal base. An alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, or heteroalkynyl substituent, for example, may be used as the spacer. Possible simple reactions for forming the covalent bond between the catalytically redox-active moiety and the nucleic acid oligomer thus modified are, as described under a), amidation from an acid and amino group, esterification from an acid and alcohol group, thioester formation from an acid and thioalcohol group, or condensation of aldehyde and amine with subsequent reduction of the resultant CH=N bond to a CH_2-NH bond. The attachment of the catalytically redox-active moiety may take place completely or in portions of the catalytically redox-active moiety with subsequent completion of the moiety (see below).

c) In the case of catalytically redox-active moieties having FAD/$FADH_2$ as cofactors, in synthesizing the nucleic acid oligomer, a phosphorylated adenine is used as the terminal base and is modified by fusion with flavin mononucleotide to a FAD derivative (β-D-2-deoxyribose-FAD) and the catalytically redox-active moiety is completed by reconstitution with the catalytically redox-active moiety that has been freed of (a) FAD.

d) If NAD^+/NADH-dependent enzymes are used (enzymes comprising cofactor(s) and apoprotein(s) and requiring, to complete a course of the catalytic reaction cycle, in addition to the specific substrate, NAD^+ or NADH, such as lactate dehydrogenase (LDH) or alcohol dehydrogenase (ADH)),

the catalytically redox-active moiety (e.g. the LDH or ADH) may be attached to the nucleic acid oligomer by covalently binding (modified) NAD^+ to the nucleic acid oligomer directly (as described here under (a)) or via a spacer (as described here under (b) or in Example 3) and associating the NAD^+-dependent enzyme with the (modified) NAD^+ through non-covalent interaction.

e) In synthesizing the nucleic acid oligomer, a terminal base or a terminal nucleotide is replaced by a cofactor of the catalytically redox-active moiety and the catalytically redox-active moiety is completed by reconstitution with the catalytically redox-active moiety that has been freed of this cofactor (see below).

[047] According to the present invention, the binding of the catalytically redox-active moiety to the nucleic acid oligomer may take place completely or in portions, before or after the nucleic acid oligomer is bound to the conductive surface. Thus, in the case of a redox-active protein/enzyme comprising apoprotein and cofactor(s), instead of the complete catalytically redox-active moiety, it is also possible for only the apoprotein, the apoprotein and a portion of the cofactors, or one or more cofactors to be attached and the catalytically redox-active moiety will be completed by subsequent reconstitution with the remaining missing portions.

[048] If there are multiple different nucleic acid oligomer combinations (test sites) on an electrode array, and the catalytically redox-active moiety is intended to be attached to the surface after the nucleic acid oligomer is immobilized, it is advantageous to standardize the (covalent) attachment of the catalytically redox-active moiety to the nucleic acid oligomers for the entire surface by the appropriate choice of reactive group at the free nucleic acid oligomer ends of the various electrodes/test sites.

[049] If redox-active proteins/enzymes are used as the catalytically redox-active moiety, the covalent attachment of the nucleic acid oligomer may take place at any reactive group that is naturally present on or affixed to the protein by modification, or – in the event that the redox-active protein/enzyme consists of apoprotein and cofactor(s) – at any reactive group that is naturally present on or affixed to a (any)

cofactor by modification. In the context of the present invention, the covalent attachment at any reactive group that is naturally present on or affixed by modification to a (any) cofactor of the protein is preferred. Without wanting to be bound to mechanistic details, if there are multiple cofactors, special preference is given to the one that can give up electrons to an external oxidizing agent that is likewise covalently attached to the nucleic acid oligomer, or that can take up electrons from an external reducing agent that is likewise covalently attached to the nucleic acid oligomer (see also the section "Method of Amperometrically Detecting Nucleic Acid Oligomer Hybrids").

The Conductive Surface

[050] According to the present invention, the term "conductive surface" is understood to mean any support having an electrically conductive surface of any thickness, especially surfaces comprising platinum, palladium, gold, cadmium, mercury, nickel, zinc, carbon, silver, copper, iron, lead, aluminum, and manganese.

[051] In addition, any doped or non-doped semiconductor surfaces of any thickness may also be used. All semiconductors may be used in the form of pure substances or in the form of mixtures. Examples include, but are not limited to, carbon, silicon, germanium, α tin, and Cu(I) and Ag(I) halides of any crystal structure. Also suitable are all binary compounds of any composition and any structure comprising the elements of groups 14 and 16, the elements of groups 13 and 15, and the elements of groups 15 and 16. In addition, ternary compounds of any composition and any structure comprising the elements of groups 11, 13, and 16 or the elements of groups 12, 13, and 16 may be used. The designations of the groups of the periodic system refer to the IUPAC recommendation of 1985.

Binding a Nucleic Acid Oligomer to the Conductive Surface

[052] According to the present invention, a nucleic acid oligomer is linked directly or via a linker/spacer with the surface atoms or molecules of a conductive surface of the type described above. This binding may be carried out in three different ways:

a) The surface is modified in such a way that a reactive molecule group is accessible. This may take place by direct derivatization of the surface molecules, for example by wet chemical or electrochemical oxidation/reduction. Thus, for example, the surface of graphite electrodes can be provided with aldehyde or carboxylic-acid groups by wet chemical oxidation. Electrochemically, it is possible, for example by reduction in the presence of aryl-diazonium salts, to couple the appropriate (functionalized, i.e., provided with a reactive group) aryl radical, or by oxidation in the presence of $R'CO_2H$, to couple the (functionalized) R'-radical to the graphite electrode surface. An example of direct modification of semiconductor surfaces is the derivatization of silicon surfaces to reactive silanols, i.e. silicon supports having Si-OR" groups on the surface, both R" and R' representing any functionalized organic residue (e.g. alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, or heteroalkynyl substituent). Alternatively, the entire surface may be modified by covalently attaching a reactive group of a bifunctional linker, such that a monomolecular layer comprising any molecules and including a reactive group, preferably terminally, results on the surface. The term "bifunctional linker" is understood to mean any molecule of any chain length, especially chain lengths 2 - 14, having two identical (homobifunctional) or two different (heterobifunctional) reactive molecule groups.

[053] If multiple different test sites are to be formed on the surface by making use of the methodology of photolithography, then at least one of the reactive groups of the homo- or heterobifunctional linkers is a photoinducibly reactive group, i.e., a group that becomes reactive only upon irradiation with light of a specific or any given wavelength. This linker is applied in such a way that the/a photoactivatable reactive group is available after the linker is covalently attached to the surface. The nucleic acid oligomers are covalently attached to the surface thus modified, and are themselves modified with a reactive group via a spacer of any composition and chain length, especially a chain length of 1 - 14, preferably near an end of the nucleic acid oligomer. The reactive group of the oligonucleotide is one of any of the groups that react directly (or indirectly) with the modified surface to form a covalent bond. In addition, a further reactive group may be bound to the nucleic acid

oligomers near their second end, this reactive group, in turn, being attached, as described above, directly or via a spacer of any composition and chain length, especially a chain length of 1 - 14. Furthermore, as an alternative to this further reactive group, the catalytically redox-active moiety (completely or portions thereof) may be attached to this second end of the nucleic acid oligomer.

b) The nucleic acid oligomer that is to be applied to the conductive surface is modified with one or more reactive groups via a covalently-attached spacer of any composition and chain length, especially a chain length of 1 - 14, the reactive groups being located preferably near an end of the nucleic acid oligomer. The reactive groups are groups that can react directly with the unmodified surface. Some examples are: (i) thiol- (HS-) or disulfide- (S-S-) derivatized nucleic acid oligomers having the general formula (n x HS-spacer)-oligo, (n x R-S-S-spacer)-oligo, or oligo-spacer-S-S-spacer-oligo that react with a gold surface to form gold-sulfur bonds or (ii) amines that attach to platinum or silicon surfaces by chemisorption or physisorption. In addition, a further reactive group may be bound to the nucleic acid oligomers near their second end, this reactive group, in turn, being attached, as described above, directly or via a spacer of any composition and chain length, especially a chain length of 1 - 14. Furthermore, as an alternative to this further reactive group, the catalytically redox-active moiety (completely or portions thereof) may be attached at this second end of the oligonucleotide. Particularly nucleic acid oligomers that are modified with multiple spacer-bridged thiol or disulfide bridges ((n x HS-spacer)-oligo or (n x R-S-S-spacer)-oligo) have the advantage that such nucleic acid oligomers can be applied to the conductive surface at a specific setting angle (angle between the surface normal and the helix axis of a double-stranded helical nucleic acid oligomer or between the surface normal and the axis perpendicular to the base pairs of a double-stranded non-helical nucleic acid oligomer) if the spacers attaching the thiol or disulfide functions to the nucleic acid oligomer possess an increasing or decreasing chain length as viewed from an end of the nucleic acid.

c) Groups used as the reactive group on the probe nucleic acid oligomer are phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine groups of the oligonucleotide backbone, especially terminal groups. The phosphoric-acid,

sugar-C-3-hydroxy, carboxylic-acid, or amine groups exhibit increased reactivity and consequently easily undergo typical reactions such as amidation with (primary or secondary) amino or acid groups, esterification with (primary, secondary, or tertiary) alcohols or acid groups, thioester formation with (primary, secondary, or tertiary) thioalcohols or acid groups, or condensation of amine and aldehyde with subsequent reduction of the resultant CH=N bond to a CH_2-NH bond. In this case, the coupling group required for covalent attachment to the phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine group is part of the surface derivatization with a (monomolecular) layer having any molecule length, as described under a) in this section, or the phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine group can react directly with the unmodified surface, as described under b) in this section. In addition, a further reactive group may be bound to the oligonucleotides near their second end, this reactive group, in turn, being attached, as described above, directly or via a spacer of any composition and chain length, especially a chain length of 1 - 14. Furthermore, as an alternative to this further reactive group, the catalytically redox-active moiety (completely or portions thereof) may be attached to this second end of the nucleic acid oligomer.

[054] Binding the nucleic acid oligomer to the conductive surface may take place before or after the catalytically redox-active moiety is attached to the nucleic acid oligomer. In the case of a redox-active protein/enzyme comprising apoprotein and cofactor(s), instead of the complete catalytically redox-active moiety, it is also possible for only the apoprotein, the apoprotein and a portion of the cofactors, or one or more of the cofactors to be attached and the catalytically redox-active moiety will be completed by subsequent reconstitution with the remaining missing portions. If a linked (at least bimolecular) electron-donor/electron-acceptor complex is used as the redox-active moiety, the electron acceptor (or donor) may, as described under b) or c) in the section "Binding a Catalytically Redox-Active Moiety to a Nucleic Acid Oligomer," be attached to a terminal base, or in place of a terminal base, to the nucleic acid oligomer, and the electron donor (or acceptor) may be attached by subsequent covalent attachment to a reactive group of the electron acceptor (or donor) or, as described under a) in the section "Binding a Catalytically Redox-Active Moiety to a Nucleic Acid Oligomer," by subsequent attachment to a terminal reactive group of the nucleic acid oligomer backbone at

the same end (see also the section "Manner of Executing the Invention"). Alternatively, binding the nucleic acid oligomer to the conductive surface may take place before or after the spacer having a reactive group for binding the catalytically redox-active moiety is attached. Binding the already modified nucleic acid oligomer to the conductive surface, i.e. binding to the surface after the catalytically redox-active moiety is attached to the nucleic acid oligomer or after portions of the catalytically redox-active moiety are attached, or after the spacer having a reactive group for binding the catalytically redox-active moiety is attached, likewise takes place as described under a) to c) in this section.

[055] In producing the test sites, when attaching the single-strand nucleic acid oligomers to the surface, care must be taken that sufficient distance remains between the individual nucleic acid oligomers to provide, first, the space necessary for hybridization with the target nucleic acid oligomer and, second, the space necessary for the attachment of the catalytically redox-active moiety. Three different methods of proceeding (and combinations thereof) offer themselves for this purpose:

1.) Producing a modified surface by attaching a hybridized nucleic acid oligomer, in other words a surface derivatization with hybridized probe nucleic acid oligomer instead of with single-strand probe oligonucleotide. The nucleic acid oligomer strand used for hybridization is unmodified (the surface attachment is carried out as described under a) - c) in this section). Thereafter, the hybridized nucleic acid oligomer double-strand is thermally dehybridized, thus producing a single-strand-nucleic-acid-oligomer-modified surface having greater distance between the probe nucleic acid oligomers.

2.) Producing a modified surface by attaching a single-strand or double-strand nucleic acid oligomer, adding, during surface derivatization, a suitable monofunctional linker that, in addition to the single-strand or double-strand nucleic acid oligomer, is likewise bound to the surface (the surface attachment is carried out as described under a) - c) in this section). According to the present invention, the monofunctional linker has a chain length that is identical to the chain length of the spacer between the surface and the nucleic acid oligomer, or that differs by a maximum of four chain atoms. If double-strand nucleic acid oligomer is used for surface derivatization, the nucleic acid oligomer double-strand is thermally

dehybridized after the double-strand nucleic acid oligomer and the linker are jointly attached to the surface. By simultaneously attaching a linker to the surface, the distance between the single-strand or double-strand nucleic acid oligomers that are likewise bound to the surface is increased. If a double-strand nucleic acid oligomer is used, this effect is amplified further by the subsequent thermal dehybridization.

3.) Producing a modified surface by attaching a single-strand or double-strand oligonucleotide to which the catalytically redox-active moiety is already attached, the catalytically redox-active moiety having a diameter of greater than 30 Å. If double-strand oligonucleotide is used, the oligonucleotide double-strand is thermally dehybridized after the double-strand oligonucleotide is attached to the surface.

[056] Regarding the individual steps in "Binding a Catalytically Redox-Active Moiety to a Nucleic Acid Oligomer," as well as in "Binding an Oligonucleotide to the Conductive Surface," it should be noted that, in the section "Manner of Executing the Invention," the various combination possibilities of the individual steps that lead to the same end result are demonstrated in an example (Fig. 2).

Method of Electrochemically Detecting Nucleic Acid Oligomer Hybrids

[057] Advantageously, according to the method of electrochemically detecting nucleic acid oligomer hybrids, multiple probe nucleic acid oligomers varying in sequence, and for de novo sequencing, ideally all necessary combinations of the nucleic acid oligomer, are applied to an oligomer (DNA) chip to detect the sequence of any target nucleic acid oligomer or (fragmented) target DNA, or in order to seek and sequence-specifically detect mutations in the target, or in order to detect the presence of known genes or known nucleic acid oligomers.

[058] For this purpose, an array is used comprising microelectrodes that are connected individually and directly to a current/voltage source, or an electrode array in which the individual electrodes may be targeted and read out through cMOS technology is applied to a common surface by microstructuring. On the conductive surface of the individual electrodes (of a test site), the surface atoms or molecules are linked with DNA/RNA/PNA nucleic acid oligomers having a known but arbitrary sequence, as described above. In a most general embodiment, however, a single electrode also may be derivatized with a single probe

oligonucleotide or a single type of probe oligonucleotide (having the same base sequence and the same catalytically redox-active moiety). Preferred probe nucleic acid oligomers are nucleic acid oligomers (e.g. DNA, RNA, or PNA fragments) of base length 3 to 50, preferably of length 5 to 30, particularly preferably of length 8 to 25. According to the present invention, a catalytically redox-active moiety is or becomes bound to the probe nucleic acid oligomers, as described below.

[059] Furthermore, parallel detection of hybridization events may also be achieved either by using various catalytically redox-active moieties for the individual electrodes of the array when forming the various functionalized electrodes of an electrode array or by using a continuously conductive surface to form the functionalized electrodes and by achieving the differentiability of molecular structures on a specific area having an identical electrode structure (to that of a specific test site) within the entire system (of the complete oligomer chip) by using, for the individual test sites, various catalytically redox-active moieties that can be addressed by selectively adding the relevant specific substrate. In the latter variant, the electrochemical response of the entire oligomer chip is detected as a result of the continuous conductive surface; addressing and reading out the electrochemical response of individual test sites occurs by selectively adding the relevant specific substrate for this test site.

[060] The modification of the probe nucleic acid oligomers with a catalytically redox-active moiety may take place completely or in components of the catalytically redox-active moiety, either before or after the oligonucleotide probe is bound to the conductive surface. The various combination possibilities of the individual steps (reaction sequences) are demonstrated in the section "Manner of Executing the Invention" with the aid of Fig. 2 using the example of a catalytically redox-active moiety bound to an electrode via a probe oligonucleotide.

[061] Regardless of the respective reaction sequence, a surface hybrid having the general structure elec-spacer-ss-oligo-spacer-moiety results, "moiety" representing the catalytically redox-active moiety. The bridges may, of course, also be produced without spacers or with only one spacer (elec-ss-oligo-spacer-moiety or elec-spacer-ss-oligo-moiety). In the example in Fig. 2, the moiety is the glucose oxidase (GOx), a redox-active enzyme consisting of apoprotein and cofactor. In the example in Figs. 2, 3, and 4, the GOx, via its cofactor flavin adenine dinucleotide (FAD) in what is known as the FAD protein binding pocket of the GOx, is covalently joined with the nucleic acid oligomer. The GOx forms a 1:1 complex with the

cofactor FAD, the GOx occurring in its natural form as a homodimer, but also exhibiting catalytic activity as a monomer, its form that is relevant to the present invention. In the example in Figs. 5 and 6, the moiety is lactate dehydrogenase, a NAD^+-dependent enzyme, that associates with the (modified) NAD^+ that is covalently bound to the probe oligonucleotide via non-covalent interaction.

[062] The electrochemical communication between the (conductive) surface and the catalytically redox-active moiety ("moiety") bridged via a single-strand oligonucleotide having the general structure elec-spacer-ss-oligo-spacer-moiety is weak or nonexistent.

[063] In a next step, the test sites are brought into contact with the nucleic acid oligomer solution to be examined (target). This leads to hybridization only if the solution contains nucleic acid oligomer strands that are complementary to the probe nucleic acid oligomers bound to the conductive surface, or complementary in at least wide areas. Hybridization between the probe and target nucleic acid oligomers leads to increased conductivity between the surface and the catalytically redox-active moiety, since the latter is now bridged via the nucleic acid oligomer consisting of a double-strand. Fig. 3 illustrates this schematically using elec-spacer-ss-oligo-spacer-FAD(GOx) as an example. In Fig. 4, the sequence of the electron transfer steps in elec-spacer-ds-oligo-spacer-FAD(GOx) is shown in detail, while Fig. 5 schematically illustrates the example elec-spacer-ss-oligo-spacer-PQQ-NAD^+-LDH and Fig. 6 shows in detail the sequence of the electron transfer steps in elec-spacer-ds-oligo-NAD^+-LDH.

[064] As a result of the hybridization of the probe nucleic acid oligomer and the nucleic acid oligomer strand (target) that is complementary thereto, the electrical communication between the (conductive) surface and the catalytically redox-active moiety changes. Thus, a sequence-specific hybridization event can be detected by electrochemical methods such as cyclic voltammetry, amperometry, potentiometry, or conductivity measurements.

[065] In cyclic voltammetry, the potential of a stationary working electrode is changed linearly as a function of time. Starting at a potential at which no electrooxidation or electroreduction occurs, the potential is changed until the redox-active substance is oxidized or reduced (i.e., a current flows). After running through the oxidation or reduction operation, which produces in the current-voltage curve an initially increasing current, then a maximum current (peak), and finally a gradually decreasing current, the direction of the potential feed is reversed. The

behavior of the products of electrooxidation or electroreduction is then recorded in reverse.

[066] An alternative electrical detection method, amperometry, is made possible by applying a suitable constant electrode potential such that the catalytically redox-active moiety may be electrooxidized (electroreduced), but the rereduction (reoxidation) of the catalytically redox-active moiety to its original state takes place, not by changing the electrode potential as in cyclic voltammetry, but rather by adding a suitable reducing agent (oxidizing agent), the "redox-active substance," to the target solution, thereby closing the current circuit of the entire system. As long as such a reducing agent (oxidizing agent) is present, or as long as the consumed reducing agent (oxidizing agent) is rereduced (reoxidized) at the counter electrode, a current flows that can be amperometrically detected and that is proportional to the number of hybridization events.

[067] This principle of amperometric detection will be explained in greater detail using the example of glucose oxidase (cf. also Figs. 3 and 4). The probe oligonucleotide having one end covalently attached to the electrode can be functionalized at the other, free end with the complete glucose oxidase enzymatic moiety, for example by covalently attaching the flavin adenine dinucleotide (FAD) cofactor of the enzyme to the probe oligonucleotide and subsequently reconstituting it with the glucose oxidase apoprotein (GOx). The resultant surface hybrid having the general structure elec-spacer-ss-oligo-spacer-FAD(GOx) exhibits little or no conductivity between the electrode and the FAD. Hybridization with the "ss-oligo"-complementary target oligonucleotide significantly increases the conductivity. Upon adding the glucose substrate to the target oligonucleotide solution, the FAD of the glucose oxidase (FAD(GOx)) is reduced to $FADH_2$ of the glucose oxidase ($FADH_2$(GOx)), glucose being oxidized to gluconic acid. If a suitable external potential is then applied to the electrode such that electrons from $FADH_2$(GOx) are given up to the electrode via the hybridized oligonucleotide, and $FADH_2$(GOx) is thus reoxidized to FAD(GOx) (but neither glucose nor gluconic acid can be electrooxidized or electroreduced at this potential), a current will flow in the elec-spacer-ds-oligo-spacer-FAD(GOx) system as long as FAD(GOx) is reduced by free glucose, i.e. until all of the glucose is consumed or, in the event that a potential at which gluconic acid can be reduced to glucose is applied to the counter electrode, as long as gluconic acid is reduced at the counter electrode. This current

can be detected amperometrically and is proportional to the number of hybridization events.

[068] In potentiometric detection of hybridization events, the course of the electrode potential is recorded as a function of, for example, substrate consumption. Here, for example, the potential of a stationary working electrode is set to the substrate's "zero current" potential E^0. When the substrate is consumed by the catalytically redox-active moiety (in the event of hybridization), the "zero current" potential E^0 changes in the direction of the equilibrium potential E^{eq}. Thus, recording the potential as a function of time (~ substrate consumption) provides information on the hybridization state.

[069] BRIEF DESCRIPTION OF THE DRAWINGS

[070] The invention will be explained in greater detail below by reference to exemplary embodiments in association with the drawings, wherein:

[071] Fig. 1 Shows a schematic diagram of oligonucleotide sequencing by hybridization on a chip;

[072] Fig. 2 Shows various reaction sequences for producing the surface hybrid elec-spacer-ss-oligo-spacer-PQQ-FAD(GOx). The catalytically redox-active moiety in this surface hybrid is the glucose oxidase (GOx) consisting of the apoprotein and the flavin adenine dinucleotide (FAD) cofactor. The GOx, via its cofactor FAD, is covalently joined via PQQ and a spacer with the oligonucleotide;

[073] Fig. 3 Shows a schematic diagram of the amperometric measurement method using the example of the surface hybrid elec-spacer-ss-oligo-spacer-PQQ-FAD(GOx) in Fig. 2 (Inj: addition (injection) of the glucose substrate);

[074] Fig. 4 Shows a detailed schematic diagram of the surface hybrid Au-S(CH_2)$_2$-ds-oligo-spacer-FAD(GOx) of Fig. 3 having gold as the surface material, mercaptoethanol as the spacer (-S-CH_2CH_2- spacer) between the electrode and the oligonucleotide, and -CH_2-CH=CH-CO-NH-CH_2-CH_2-NH-PQQ-NH-CH_2-CH_2- as the spacer between the cofactor FAD and the oligonucleotide, as well as a diagram of the sequence of the substrate-induced electron transfer steps. The apoprotein of the GOx is indicated only as a shell (solid line) (cf. Structure 1). The 12-bp probe oligonucleotide of the exemplary sequence 5'-TAGTCGGAAGCA-3' in the hybridized state is shown in detail;

[075] Fig. 5 Shows a schematic diagram of the amperometric measurement method using the example of the surface hybrid elec-spacer-ss-oligo-spacer-PQQ-NAD^+-LDH (Inj: injection of the lactate substrate); and

[076] Fig. 6 Shows a detailed schematic diagram of the surface hybrid Au-$S(CH_2)_2$-ds-oligo-spacer-PQQ-NAD^+-LDH of Fig. 3 having gold as the surface material, mercaptoethanol as the spacer (-S-CH_2CH_2- spacer) between the electrode and the oligonucleotide, and -CH_2-CH=CH-CO-NH-CH_2-CH_2-NH-PQQ-NH-CH_2-CH_2- as the spacer between the NAD^+ and the oligonucleotide to which ADH is associated, as well as a diagram of the sequence of the substrate-induced electron transfer steps. The 12-bp probe oligonucleotide of the exemplary sequence 5'-TAGTCGGAAGCA-3' in the hybridized state is shown in detail.

[077] MANNER OF EXECUTING THE INVENTION

[078] A formation unit of an exemplary test site with hybridized target, Au-$S(CH_2)_2$-ds-oligo-spacer-PQQ-FAD(GOx) having the general structure elec-spacer-ds-oligo-spacer-moiety, is illustrated in Fig. 4. In the context of the present invention, "formation unit" is understood to mean the smallest repeating unit of a test site or functionalized electrode within the electrode array. In the example in Fig. 4, the surface is a gold electrode. The link between the gold electrode and the probe oligonucleotide was formed with the linker (HO-$(CH_2)_2$-$S)_2$, which was esterified with the terminal phosphate group at the 3'-end to form P-O-$(CH_2)_2$-S-S-$(CH_2)_2$-OH and, following homolytic cleavage of the S-S bond at the gold surface, produced one Au-S bond each, with which 2-hydroxy-mercaptoethanol and mercaptoethanol-bridged oligonucleotide was coadsorbed on the surface. The catalytically redox-active moiety in the example in Fig. 4 is the glucose oxidase (GOx), a redox-active enzyme consisting of apoprotein and FAD cofactor(s). In the application example, the GOx, via its FAD cofactor, is covalently joined with the oligonucleotide, free FAD having first been provided with a reactive amino group (see Example 1), then covalently attached to the probe oligonucleotide via this amino group (amidation and dehydration with a carboxylic-acid group of the PQQ bound with another carboxylic-acid group to the terminal amino function of the -CH=CH-CO-NH-CH_2-CH_2-NH_2 linker attached at the C-5 position of the 5' thymine of the probe oligonucleotide), and finally, the apoprotein of the GOx was reconstituted to FAD.

[079] As already mentioned above, the modification of the probe oligonucleotides may take place with the complete catalytically redox-active moiety or with a component thereof, either before or after the probe oligonucleotide is bound to the conductive surface. The various combination possibilities of the individual steps, which lead in effect to the same formation unit of a test site or functionalized electrode within the electrode array, will be illustrated below with the aid of Fig. 2 using the example of the surface hybrid Au-S$(CH_2)_2$-ss-oligo-spacer-PQQ-FAD(GOx) or its more general form, elec-spacer-ss-oligo-spacer-PQQ-FAD(GOx).

[080] The GOx can be freed of the FAD cofactor by simple manipulation (cf. Example 4), thus making it possible to break GOx down into two components, FAD and apoprotein. The probe oligonucleotide has (identical or differing) reactive groups attached, via a (any) spacer, near each of its ends. In a reaction sequence "1," in the presence of a monofunctional linker (according to points a) - c) and 2.) in the section "Binding an Oligonucleotide to the Conductive Surface"), the probe oligonucleotide thus modified may be covalently attached to the electrode together with the monofunctional linker, making sure that sufficient monofunctional linker of suitable chain length is added to provide sufficient space between the individual probe oligonucleotides to permit hybridization with the target oligonucleotide and to permit the attachment of the catalytically redox-active moiety. Thereafter, to the free spacer-bridged reactive group of the probe oligonucleotide is attached PQQ, and to that, N^6-(2-aminoethyl)-FAD (Formula 5 or Example 1). The attachment takes place as described under a) or b) in the section "Binding a Catalytically Redox-Active Moiety to a Nucleic Acid Oligomer" or in Example 4. In the last step of this reaction sequence "1," the apoprotein of the GOx is then reconstituted to the (modified) FAD cofactor, as described in Example 2. In a variation hereof (reaction sequence "2"), the modified (with spacer and reactive groups) probe oligonucleotide can first be covalently bound to the electrode without a free monofunctional linker (spacer), causing a flat attachment of the oligonucleotide. Thereafter, the free monofunctional linker (spacer) is covalently bound to the electrode. A further possibility (reaction sequence "3") is to modify the modified (with spacer and reactive groups) probe oligonucleotide first with PQQ and FAD, then covalently attach it to the electrode in the presence of a free monofunctional linker (spacer), and thereafter reconstitute it with the GOx apoprotein. Finally, in a reaction sequence "4," the modified (with spacer and reactive groups) probe

oligonucleotide can first be modified with PQQ and FAD so as then to reconstitute it with the GOx apoprotein and, thereafter, to covalently bind it to the electrode. In the event that, as in the case of the GOx, the catalytically redox-active moiety has a significantly greater diameter than the hybridized ds-oligonucleotide (greater than 30 Å), the covalent attachment of a suitable free monofunctional linker (spacer) to the electrode can be dispensed with; otherwise, the attachment of the structure -spacer-ss-oligo-spacer-PQQ-FAD(GOx) to the electrode occurs in the presence of a suitable free monofunctional linker.

[081] In the example in Fig. 2, the GOx, via its FAD-cofactor is covalently joined with the oligonucleotide. Alternatively, instead of the FAD-cofactor, the apoprotein also can be covalently attached to the probe oligonucleotide, any combinations of the reaction sequences "1," "2," "3," or "4" in Fig. 2 may be applied, as long as they yield the same end product (cf. Fig. 2), and, in any reaction steps, the probe oligonucleotide hybridized with complementary, unmodified (target) oligonucleotide may be used in place of the single-strand probe oligonucleotides. The probe oligonucleotide can also be attached directly, in other words not bridged via a spacer, to both the electrode and the catalytically redox-active moiety, as described under c) in the section "Binding a Nucleic Acid Oligomer to the Conductive Surface" or under a) in the section "Binding a Catalytically redox-active Moiety to a Nucleic Acid Oligomer."

[082] The electrical communication between the conductive surface and the catalytically redox-active moiety bridged via a single-strand oligonucleotide in the general structure elec-spacer-ss-oligo-spacer-moiety is weak or nonexistent. If hybridization occurs between the probe and the target, treating the test site(s) with an oligonucleotide solution to be examined causes increased conductivity between the surface and the catalytically redox-active moiety bridged via a double-strand oligonucleotide. For the formation unit of the test site Au-S$(CH_2)_2$-ds-oligo-spacer-PQQ-FAD(GOx) (with 12-bp probe oligonucleotides) used as an example, this is shown schematically in Fig. 3 using amperometric measurements.

[083] By adding glucose, electrons are transferred to the FAD-cofactor of the GOx. If a suitable potential is applied to the electrode to transfer electrons from the reduced FAD (FAD^- or FAD^{2-} or $FADH_2$) to the electrode, current still will not flow in the case of the probe oligonucleotide not hybridized with the target oligonucleotide, since the conductivity of the ss-oligonucleotide in Au-S$(CH_2)_2$-ss-

oligo-spacer-PQQ-FAD(GOx) is very slight or nonexistent. In the hybridized state (Au-S$(CH_2)_2$-ds-oligo-spacer-PQQ-FAD(GOx)), however, conductivity is high, electrons can be transferred from the reduced FAD (FAD^- or FAD^{2-} or $FADH_2$) to the electrode (forming FAD). This manifests itself amperometrically in a distinct flow of current between the electrode and the catalytically redox-active moiety (Fig. 3). It is thus possible to detect the sequence-specific hybridization of the target with the probe oligonucleotides by amperometry. The individual electron transfer steps that are triggered in the surface hybrid Au-S$(CH_2)_2$-ds-oligo-spacer-PQQ-FAD(GOx) by the substrate are illustrated in Fig. 4. In principle, given suitable external conditions, the surface hybrid Au-S$(CH_2)_2$-ds-oligo-spacer-PQQ-FAD(GOx) can also be reversed, such that FAD is reduced by the electrode and reduced FAD (FAD^- or FAD^{2-} or $FADH_2$) is oxidized by a suitable substrate in a catalytic reaction.

[084] A further test site or a further functionalized electrode within the electrode array, Au-S$(CH_2)_2$-ds-oligo-spacer-PQQ-NAD^+-LDH, having the general structure elec-spacer-ss-oligo-spacer-moiety, is illustrated in Fig. 5. By adding the ADH substrate lactate, electrons are transferred to the FMN cofactor of the LDH and are passed on from this reduced FMN (FMN^- or FMN^{2-} or $FMNH_2$) to NAD^+, directly or with further cofactors of the LDH participating, and finally, from the reduced NAD^+ (NAD, NAD^-, or NADH) to the electrode. In the case of the probe oligonucleotide not hybridized with the target oligonucleotide, current still will not flow between the reduced NAD^+ (NAD, NAD^-, or NADH) and the electrode, since the conductivity of the ss-oligonucleotide in Au-S$(CH_2)_2$-ss-oligo-spacer-PQQ-NAD^+-LDH is very weak or nonexistent. In the hybridized state (Au-S$(CH_2)_2$-ds-oligo-spacer-PQQ-NAD^+-LDH), however, conductivity is high, electrons can be transferred from the reduced NAD^+ (NAD, NAD^-, or NADH) to the electrode (forming NAD^+). This manifests itself amperometrically in a distinct flow of current between the electrode and the catalytically redox-active moiety (Fig. 5). The sequence-specific hybridization of the target with the probe oligonucleotides can thus be detected by amperometry. The individual electron transfer steps that are triggered in the surface hybrid Au-S$(CH_2)_2$-ds-oligo-spacer-PQQ-NAD^+-ADH by the substrate are illustrated in Fig. 6. In principle, given suitable external conditions, the surface hybrid Au-S$(CH_2)_2$-ds-oligo-spacer-PQQ-NAD^+-LDH can also be reversed, such that NAD^+ is reduced by the electrode and reduced NAD^+ (NAD, NAD^-, or NADH) is oxidized by a suitable substrate (e.g. acetaldehyde) in a catalytic reaction.

[085] Since the redox activity of the catalytically redox-active moiety – even at the appropriate electrode potential – is triggered only by adding the specific substrate and is maintained, at most, as long as the substrate is present, this condition can be used to advantage, according to the present invention, in that a specific test site or group of test sites of an oligomer chip is spatially resolved by using various catalytically redox-active moieties for the various test sites or groups of test sites. The advantage of doing so, according to the present invention, is that the various test sites (nucleic acid oligomer combinations) of an oligomer chip can be applied to a shared, continuous, electrically-conductive surface and a specific test site or a specific group of test sites can be addressed and amperometrically detected simply by adding the relevant specific substrate. The various test sites thus need not be applied to individual (micro-) electrodes that are electrically isolated from one another and individually targetable for potential application and current read-out.

[086] In addition, defective base pairings (base-pair mismatches) can be recognized by an altered cyclic voltammetric characteristic. A mismatch manifests itself in a greater potential difference between the current maximums of electroreduction and electroreoxidation (reversal of electroreduction when the potential feed direction is reversed) or electrooxidation and electrorereduction in a cyclic voltammetrically reversible electron transfer between the electrically-conducting surface and the catalytically redox-active moiety. This fact has an advantageous effect most importantly on amperometric detection because there, the current can be tested at a potential at which the perfectly hybridizing oligonucleotide target supplies significant current, but the defectively paired oligonucleotide target does not.

[087] ***Example 1:*** *Modification of the FAD to N^6-(2-aminoethyl)-FAD, Formula 5, or of the NAD^+ to N^6-(2-aminoethyl)-NAD^+*: The N^6-(2-aminoethyl)-FAD is produced by alkylating the N-1 position of the adenine moiety of FAD with aziridine (azacyclopropane) under mild, aqueous conditions (pH = 3.2 - pH = 5.5) and subsequent intramolecular Dimroth rearrangement under mild aqueous conditions (pH = 6 - 6.5, 50°C) in accordance with the specification in Bückmann et al., 1991, European Patent 0.247.537.B1. Alternatively, the method of Morris et al., Anal. Chem. 53 (1991) 658 - 665, or Zapelli et al., Eur. J. Biochem. 89 (1978) 491 - 499,

also may be applied. Under identical reaction conditions, the N^6-(2-aminoethyl)-NAD^+ can be produced, as well, if NAD^+ is used as the starting substance instead of FAD.

[088] ***Example 2:*** *Producing the oligonucleotide electrode Au-S(CH_2)$_2$-ss-oligo-spacer-PQQ-FAD(GOx)*: *Au-S(CH_2)$_2$-ss-oligo-spacer-PQQ-FAD(GOx)* is produced in 5 steps, namely producing the conductive surface, derivatizing the surface with the oligonucleotide probe in the presence of a suitable monofunctional linker (incubation step), covalently attaching the PQQ (redox step I), attaching the N^6-(2-aminoethyl)-FAD (redox step II), and reconstituting the apoprotein of the GOx (reconstitution step).

[089] In approximate 100 nm thin gold film on mica (muscovite lamina) forms the support material for the covalent attachment of the double-strand oligonucleotides. For this purpose, freshly cleaved mica was purified with an argon-ion plasma in an electrical discharge chamber and gold (99.99%) was applied by electrical discharge in a layer thickness of approx. 100 nm. Thereafter, the gold film was freed of surface impurities (oxidation of organic accumulations) with 30% H_2O_2/70% H_2SO_4 and immersed in ethanol for approx. 20 minutes to dispel any oxygen adsorbed on the surface. After rinsing the surface with bidistilled water, a previously prepared $1x10^{-4}$ molar solution of the (modified) double-strand oligonucleotide is applied on the horizontally mounted surface, such that the entire gold surface is wetted (incubation step, see also below).

[090] For incubation, a doubly modified 12-bp single-strand oligonucleotide having the sequence 5'-TAGTCGGAAGCA-3' was used, which is esterified with (HO-(CH_2)$_2$-S)$_2$ at the phosphate group of the 3'-end to form P-O-(CH_2)$_2$-S-S-(CH_2)$_2$-OH. At the 5'-end, the terminal thymine base of the oligonucleotide is modified at the C-5 carbon with -CH=CH-CO-NH-CH_2-CH_2-NH_2. Approximately 10^{-4} to 10^{-1} molar 2-hydroxy-mercaptoethanol (or another thiol or disulfide linker having a suitable chain length) was added to a $2x10^{-4}$ molar solution of this oligonucleotide in HEPES buffer (0.1 molar in water, pH 7.5 with 0.7 molar addition of TEATFB, see abbreviations) and the gold surface of a test site was completely wetted and incubated for 2-24 hours. During this reaction time, the disulfide spacer P-O-(CH_2)$_2$-S-S-(CH_2)$_2$-OH of the oligonucleotide is homolytically cleaved. In this process, the spacer forms a covalent Au-S bond with Au atoms of the surface, thus causing

a 1:1 coadsorption of the ss-oligonucleotide and the cleaved 2-hydroxy-mercaptoethanol. The free 2-hydroxy-mercaptoethanol that is also present in the incubation solution is likewise coadsorbed by forming an Au-S bond (incubation step).

[091] The gold electrode thus modified with a monolayer comprising ss-oligonucleotide and 2-hydroxy-mercaptoethanol was washed with bidistilled water and subsequently wetted with a solution of $3x10^{-3}$ molar quinone PQQ, 10^{-2} molar EDC, and 10^{-2} molar sulfo-NHS in HEPES buffer. After a reaction time of approx. 1 - 4 hours, the -CH=CH-CO-NH-CH_2-CH_2-NH_2 spacer and the PQQ form a covalent bond (amidation between the amino group of the spacer and the C-7-carboxylic-acid function of the PQQ, redox step I).

[092] Thereafter, the gold electrode thus modified was washed with bidistilled water and subsequently wetted with a solution of 1-10 x 10^{-3} molar quinone N^6-(2-aminoethyl)-FAD, 1-5 x 10^{-2} molar EDC, and 10^{-2} molar sulfo-NHS in HEPES buffer. After a reaction time of approx. 1 - 4 hours, the PQQ and the N^6-(2-aminoethyl)-FAD form a covalent bond (amidation between the amino group of the spacer and the C-2-carboxylic-acid function of the PQQ bound to the oligonucleotide, redox step II).

[093] Finally, the gold electrode thus modified was washed with bidistilled water and incubated for approx. 4 hours with a solution of approx. $5x10^{-5}$ molar FAD-free GOx in 100 mM phosphate buffer, pH = 7, with 0.5 molar addition of TEATFB at approx. 25°C, and thereafter approx. 12 hours at 4°C, to reconstitute the apoprotein of the GOx to the N^6-(2-aminoethyl)-FAD bound to the oligonucleotide and, in completion, washed with approx. 4°C-cold buffer solution (100 mM phosphate, pH = 7 with 0.5 molar addition of TEATFB) (reconstitution step).

[094] ***Example 3:*** *Producing the oligonucleotide electrode Au-S(CH_2)$_2$-ss-oligo-spacer-PQQ-NAD$^+$-LDH: Au-S(CH_2)$_2$-ss-oligo-spacer-PQQ-NAD$^+$-LDH* is produced in 5 steps, namely producing the conductive surface, derivatizing the surface with the probe oligonucleotide in the presence of a suitable monofunctional linker (incubation step), covalently attaching the PQQ (redox step I), attaching the N^6-(2-aminoethyl)-NAD^+ (redox step II), and the association with LDH (association step).

[095] An approximate 100 nm thin gold film on mica (muscovite lamina) forms the support material for the covalent attachment of the double-strand oligonucleotides.

For this purpose, freshly cleaved mica was purified with an argon-ion plasma in an electrical discharge chamber and gold (99.99%) was applied by electrical discharge in a layer thickness of approx. 100 nm. Thereafter, the gold film was freed of surface impurities (oxidation of organic accumulations) with 30% H_2O_2/70% H_2SO_4 and immersed in ethanol for approx. 20 minutes to dispel any oxygen adsorbed on the surface. After rinsing the surface with bidistilled water, a previously prepared $1x10^{-4}$ molar solution of the (modified) double-strand oligonucleotide is applied on the horizontally mounted surface, such that the entire gold surface is wetted (incubation step, see also below).

[096] For incubation, a doubly modified 12-bp single-strand oligonucleotide having the sequence 5'-TAGTCGGAAGCA-3' was used, which is esterified with $(HO-(CH_2)_2-S)_2$ at the phosphate group of the 3'-end to form $P-O-(CH_2)_2-S-S-(CH_2)_2-OH$. At the 5'-end, the terminal thymine base of the oligonucleotide is modified at the C-5 carbon with $-CH=CH-CO-NH-CH_2-CH_2-NH_2$. Approximately 10^{-4} to 10^{-1} molar 2-hydroxy-mercaptoethanol (or another thiol or disulfide linker having a suitable chain length) was added to a $2x10^{-4}$ molar solution of this oligonucleotide in HEPES buffer (0.1 molar in water, pH 7.5 with 0.7 molar addition of TEATFB, see abbreviations) and the gold surface of a test site was completely wetted and incubated for 2-24 hours. During this reaction time, the disulfide spacer $P-O-(CH_2)_2-S-S-(CH_2)_2-OH$ of the oligonucleotide is homolytically cleaved. In this process, the spacer forms a covalent Au-S bond with Au atoms of the surface, thus causing a 1:1 coadsorption of the ss-oligonucleotide and the cleaved 2-hydroxy-mercaptoethanol. The free 2-hydroxy-mercaptoethanol that is also present in the incubation solution is likewise coadsorbed by forming an Au-S bond (incubation step).

[097] The gold electrode thus modified with a monolayer comprising ss-oligonucleotide and 2-hydroxy-mercaptoethanol was washed with bidistilled water and subsequently wetted with a solution of $3x10^{-3}$ molar quinone PQQ, 10^{-2} molar EDC, and 10^{-2} molar sulfo-NHS in HEPES buffer. After a reaction time of approx. 1 - 4 hours, the $-CH=CH-CO-NH-CH_2-CH_2-NH_2$ spacer and the PQQ form a covalent bond (amidation between the amino group of the spacer and the C-7-carboxylic-acid function of the PQQ, redox step I).

[098] Thereafter, the gold electrode thus modified was washed with bidistilled water and subsequently wetted with a solution of 1-10 x 10^{-3} molar quinone N^6-

(2-aminoethyl)-NAD^+, 1-5 x 10^{-2} molar EDC, and 10^{-2} molar sulfo-NHS in HEPES buffer. After a reaction time of approx. 1 - 4 hours, the PQQ and the N^6-(2-aminoethyl)-NAD^+ form a covalent bond (amidation between the amino group of the spacer and the C-2-carboxylic-acid function of the PQQ bound to the oligonucleotide, redox step II).

[099] Finally, the gold electrode thus modified was washed with bidistilled water and incubated for approx. 1 hour with a solution of LDH (5 mg/mL) in 10 mM Tris, pH = 7, with 0.7 molar addition of TEATFB at approx. 4°C, to associate LDH to the N^6-(2-aminoethyl)-NAD^+ bound to the oligonucleotide and, in completion, washed with approx. 4°C-cold buffer solution (10 mM Tris, pH = 7 with 0.7 molar addition of TEATFB) (association step).

[100] ***Example 4:*** *Producing the apoprotein of glucose oxidase/extracting the FAD from glucose oxidase:* Glucose oxidase (GOx) in a phosphate buffer (80 mg GOx in 14 mL buffer) with a 6-mL glycerin addition is cooled to -4°C in a salt/ice bath, stirred vigorously, gradually adding 2.5% H_2SO_4 (v/v) until the pH value drops to pH = 1.4, and incubated on the ice bath in this condition for 2.5 hours. For this purpose, a sephadex G-50 column with 30% glycerin in water (v/v) is equilibrated and brought to pH = 1.4 with conc. H_2SO_4, cooled to 4°C, and applied to the incubation solution. The protein peak is eluted at a flow rate of 1.3 mL/min. and collected in a storage vessel with 0.4 molar phosphate buffer (4 mL with 200 mg of bovine serum albumin and 400 mg of activated carbon added) (the protein peak can be recognized by UV absorption of the eluate). The protein-containing eluate is set to pH = 7 and the activated carbon is removed by filtration (0.8 μm and, subsequently, 0.22 μm millipore filter). Finally, 10% sodium azide in water (w/v) is added until the total concentration of the sodium azide is 0.1% (w/v).